Veterans
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Grants Administration
Defense Technical Information Center
DTIC-O, 8725 John J. Kingman Road
Fort Belvoir, VA 22060-6218

Re: Final Technical Report - Dr. Dewleen Baker, MD – N62645-11-C-4037

Dear Grants Administration:

Please find attached two copies of the final technical report of award number N62645-11-C-4037 under the direction of Dr. Dewleen Baker. Please contact us should you require additional information or have any questions.

Sincerely,

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AD_____

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CONTRACTING ORGANIZATION: Naval Medical Logistics Command
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14. ABSTRACT

Marine Resiliency Study II (MRS II) is a collaborative project with an overarching objective to develop a platform to provide an early analysis of predictors of mental health outcomes, such as Post Traumatic Stress in Marines, in coordination with the Army Study of Risk and Resilience (Army STARRS) program, by evaluating the physical, family, social, cognitive and mental health status of ground combat Marines deploying to Iraq and Afghanistan. The three components of MRS II will provide for: 1) Extended evaluation and additional follow-up assessments of remaining active duty Marines in battalions previously assessed by Marine Resiliency Study I (MRS I). 2) A MRS II specific assessment of Marines in battalions slated for deployment to enable the use of novel, stand-alone pre- and post- deployment measures independent of other ongoing projects, but can be designed to coordinate with measures used in the ARMY STARRS. 3) Pilot/Demonstration projects based on the data from the original and MRS II study for a series of small studies to investigate the feasibility of targeted prevention or intervention protocols, or the use of new technologies to identify biomarkers. The MRS II study has completed the data collection and cleaning phase for all projects, and has included all published MRS-II manuscripts in this report. Additionally MRS-II researchers continue to work on additional analyses, which will be provided to HQMC when they are completed and accepted for publication. MRS-II researchers continue to collaborate with Army STARRS researchers on replication various analyses, including gene expression, epigenetic and genetic analyses.

15. SUBJECT TERMS
Stress, Post Traumatic Stress Disorder, Biomarker, Suicide, Combat

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Table of Contents

Introduction:

Marine Resiliency Study II (MRS II) is a collaborative project with an overarching objective to develop a platform to provide early analysis of predictors of mental health outcomes, such as Post Traumatic Stress in Marines, in coordination with the Army Study of Risk and Resilience (Army STARRS) program, by evaluating the physical, family, social, cognitive and mental health status of ground combat Marines deploying to Iraq and Afghanistan. They study aims to achieve these objectives through three main components. The three components of MRS II will provide for: 1) Extended evaluation and additional follow-up assessments of remaining active duty Marines in battalions previously assessed by Marine Resiliency Study I (MRS I), a prospective, longitudinal assessment of 2,500 Marines pre- and post-deployment across psychological, physiological and biological domains. 2) A MRS II specific assessment of Marines in battalions slated for deployment to enable the use of novel, stand-alone pre- and post- deployment measures independent of other ongoing projects, but can be designed to coordinate with measures used in the United States Army's Study to Assess Risk and Resilience in Service members (STARRS). 3) Pilot/Demonstration projects based on the data from the original and MRS II study for a series of small studies to investigate the feasibility of targeted prevention or intervention protocols, or the use of new technologies to identify biomarkers. A specific goal of these small studies would be to determine feasibility and to estimate effect sizes from more extensive studies in larger Department of Defense groups, such as that from Army STARRS.

Body:

The primary programmatic aim of the Marine Resiliency II (MRS II) is to identify the individual, social, and deployment factors that predict trajectories of mental health response, particularly posttraumatic stress disorder (PTSD), and secondly, by integrating and analyzing data across psychosocial, physiological and biological domains, to accomplish a broader multi-system understanding of the phenomenology of adaptation to stress.

The specific objectives of this collaboration and implementation of MRS II are to:

1. Provide extended assessment information from Marines already enrolled in MRS I so as to more accurately identify modifiable stressors for HQMC that most strongly predict mental health and functional outcomes.
2. In coordination with HQMC, NIMH and Army STARRS, to provide longitudinal psychosocial and biological data collection and analyses so as to better understand causes and risk of suicide across services.
3. In coordination with HQMC, NIMH and Army STARRS, to explore modifiable psychophysiological, biological and genetic biomarkers predictive of post-deployment mental health outcomes.
4. In coordination with HQMC, NIMH and Army STARRS, to test the feasibility of specific experimental designs such as targeted prevention or treatment protocols, or the use of new technology (e.g. MEG).
5. In coordination with HQMC, NIMH and Army STARRS, to determine feasibility and estimate effect sizes of experimental designs in smaller Marine cohorts for more extensive studies using the Army STARRS cohort

Task 1: To extend the number of assessment time-points and the evaluation period for all available Marines participating in the Marine Resiliency Study (MRS). Marines available include Marines from Cohort 3 (300 Marines from 3/4, 29 Palms) and Cohort 4 (400 Marines from 3/5 and 1st CEB, Pendleton). Regulatory approval (University of California (UCSD), Veterans Affairs (VA) and Naval Health Research Center (NHRC)) is current: IRB # 070533.

Task 1a: Cohort 3 extended post-deployment data collection

- Subtask 1: Organize and maintain materials for data collection
- Subtask 2: Data collection
- Subtask 3: Data entry and cleaning
- Subtask 4: Data integration and analysis

Task 1b: Cohort 4 extended post-deployment data collection

- Subtask 1: Organize materials for data collection
- Subtask 2: Data collection
- Subtask 3: Data entry and cleaning
- Subtask 4: Data integration and analysis

Current status of Task 1a and 1b:

Task 1a: Data gathering for MRS-II Project 1, (Extension Study), is complete, and data entry and cleaning have also been completed. 97 subjects were successfully enrolled into this part of the project in January of 2012. Primary analysis for this task has been completed and data has been reported in manuscripts. (subtask 4).

Task 1b: Assessments for MRS-II, Project 1, (Extension Study) were not collected, since 3/5 and 1st CEB unit did not go on a deployment.

Summary of Task 1a and 1b: All IRB approvals (UCSD, VA, and NHRC) for MRS-II, Project 1,(Extension) remain current and Core analysis has been completed reported in manuscripts. Additional ancillary analysis is ongoing and all papers and data will be shared with NMLC and Marine Corps Headquarters.

Task 2: To identify biomarker predictors of PTSD and suicide vulnerability in newly enrolled Marines, pre- and post- deployment, using measures that can be designed to coordinate with those used in Army STARRS. Specifics of these experimental designs will be developed in concert with NIMH, HQMC and Army STARRS.

These tasks are dictated by DOD and USMC leadership in that the dates that the project can be executed based on deployment and training dates of currently participating battalions and potential battalions. Data collection timeframes are subject to change based on these schedules.

Task 2a: Novel pre-post-deployment research design, coordinated with Army STARRS, 1st battalion

- Subtask 1: Development of experimental design
- Subtask 2: Regulatory approval; amendment or new IRB
- Subtask 3: Data collection
- Subtask 4: Data entry and cleaning
- Subtask 5: Data integration and analysis

Task 2b: Novel post-deployment research design, coordination with Army STARRS, 2nd battalion

- Subtask 1: Development of experimental design
- Subtask 2: Regulatory approval; amendment or new IRB
- Subtask 3: Data collection
- Subtask 4: Data entry and cleaning
- Subtask 5: Data integration and analysis

Current status of Task 2a and 2b:

Task 2a: For MRS-II, Project 2a (Neuro-cognition Study) deploying Marines from 1/7 were assessed during the allotted 2012 timeframe. In total 570 Marines were available and consented with cohort 1 (Task 2a). Seven Marines declined study participation. Of enrolled Marines, 569 completed the pre-deployment study assessments. 428 Marines completed their post-deployment assessment for a total retention rate of 75.1% of all subjects participating in this portion of the study.

Task 2b: The MRS-II military liaison and MRS staff identified a second battalion of deploying Marines (2/7) to complete study recruitment goals. Assessments of these deploying Marines took place at 29 Palms Marine Base. 625 Marines were available and consented. 34 Marines declined study participation. Of enrolled Marines, 458 Marines completed their post-deployment assessment for a total retention rate of 73.3% of all subjects participating in this portion of the study.

Summary of Tasks 2a and 2b: Thus total enrollment for Project 2 wass 1195 recruited, 41 declined and 1190 completions of the pre-deployment assessments. 886 Marines completed their post-deployment, for an overall retention rate of 75.5% for this portion of the project. A small recruitment of non-deployed subjects (3/7) was completed with 3/7 with a total enrollment of 195 recruited, 2 declined and 195 completions of the pre-deployment assessments. 163 Marines from this group completed their post-deployment, for an overall retention rate of 83.6%. Our goal in recruiting and assessing non-deploying Marines served as a non-deployment control group. All IRB approvals (UCSD, VA, and NHRC) for MRS-II, Project 2 (Neuro-cognition Study) remain current, as necessary for the data collection as noted above.

Task 3: In coordination with HQMC, NIMH and Army STARRS, implement demonstration projects that utilize subgroups drawn from prior MRS enrollees, to test the feasibility of specific experimental designs such as targeted prevention or treatment protocols or the use of new technology (e.g. MEG) to identify biomarkers. A *specific* goal of the demonstration projects would be to determine feasibility and estimate effect sizes for more extensive studies using the Army STARRS cohort.

- Subtask 1: Identification of goals of exploratory, demonstration projects
- Subtask 2: Development of experimental design and local review
- Subtask 3: Project review by NIMH, Marine Corps
- Subtask 4: Regulatory approval; amendment or new IRB
- Subtask 5: Data collection
- Subtask 6: Data entry and cleaning
- Subtask 7: Data integration and analysis

Current status of Task 3 during this reporting period:

MRS II, Project 3 had four demonstration projects:

1. For MRS-II, Project 3, Demonstration Project 1, IRB approvals remain current to allow for continued secondary analysis and manuscript writing. This project involved collaboration with University of Pennsylvania researchers associated with the Army STARRS Program. Data collection on this initial Demonstration Project focused on validation of the Penn battery (Ruben Gur) for use in Project 2 is complete (45 subjects) and all primary analysis is complete. All data analysis for this project has been completed. The wealth of data generated will also serve as a great resource for possible future data analysis, research studies, and publications.

2. For MRS-II, Project 3, Demonstration Project 2, IRB approvals are up-to-date to allow for continued secondary analysis and manuscript writing. Project samples were analyzed, and basic characterization of comparison groups (PTSD versus healthy) is complete. A total of 64 PTSD cases and 64 combat-exposed healthy, matched subjects were selected. Blood samples of these 128 Marines were available at pre-deployment and at least once at post-deployment. A total of 360 RNA and matching DNA samples have been extracted, quantified, and RNA seq is complete. The findings have been published (Breen et al., 2015). DNA samples for methylation assays (epigenetics) were submitted, and are complete. Secondary data analysis continues; one manuscript is near completion and will be submitted for review to HQMC when complete, i.e. findings are replicated by an independent data set. MRS-II is working with other prospective studies to confirm replications.

3. For MRS-II, Project 3, Demonstration Project 3, IRB approvals remain current to allow for continued secondary analysis and manuscript writing. The metabolomic measurements of all 120 samples were assayed, and primary analysis is complete. Outcomes on the first 40 Marines were presented at a Military Biomarker Workshop in Amsterdam. Validation assays were completed and analyzed. These assays were delayed because of a break-down in equipment, but were ultimately completed. Initial analysis indicates that groups (PTSD, mTBI, PTSD/mTBI, control) can be grouped by metabolomic profile. The number of groupings was larger than anticipated. Additional samples will be needed for analysis as a separate project. DOD funds are currently being sought to complete additional assays and subsequent analysis.

4. For MRS-II, Project 3, Demonstration Project 4, all projects IRB approvals remain current to allow for continued secondary analysis and manuscript writing. This project was reviewed and approved by NIMH and HQMC. Funding for this study was allocated and the project was reviewed and approved by both the Naval Health Research Center IRB and the VA-designated IRB: UCSD Human Research Protections Program. With the help of our POC at HQMC, John Hartmann, an

   EOD battalion was identified to participate and their post deployment and post training assessments (December 2014) were completed. An initial group of fifty Marines signed the informed consents during the June time frame (Jun 12 and Jun. 14, 2013 and Jun 26, 2013), and an additional 20 signed informed consents shortly thereafter. Enrolled Marines completed baseline scans and underwent blast training. Post-training scans took place in fall 2014 to summer of 2015. Preliminary outcomes of baseline assessments were presented at the Military Biomarker Workshop in Amsterdam. Additionally, IRB approval (an adjunct protocol) was obtained to proceed with distribution of blast sensors, and to analyze MEG data in relation to blast event outcomes during field training. Of the enrolled Marines in the Demonstration Project 4: MEG Study we obtained consents for use of the blast sensors from 60 Marines during the allotted October to December 2013 timeframe. Of consented Marines, 54 (77.1%) received their blast sensors. Blast sensors from these Marines were collected and core analysis is complete. Additionally, IRB approval (an adjunct protocol) was obtained to proceed with distribution of blast sensors during deployment. Of the enrolled Marines in the Demonstration Project 4: MEG Study we obtained consent for use of the blast sensors from 17 Marines during the allotted March 2014 timeframe. A subset of the EOD battalion went on deployment and 17 (24.2%) have received blast sensors which were collected upon their return.

   Upon receiving notification that the EOD battalion returned to stateside we began working with the battalion leadership to schedule assessments. Due to post deployment operational requirements (OPTEMPO) for this unit, Marines were available for post-deployment assessments later that originally projected, thus our data collection schedule was delayed approximately 6 months. In November and December, 2014 we completed our first post deployment assessment session and assessed 9 subjects who were available. Our second assessment session began January of 2015; we completed assessment of 11 Marines as of March 2015. Our third assessment session began April of 2015; but due to operational requirements of Marines needing scans we were able to assess one Marine during this timeframe.

   A fourth assessment session began July of 2015 to assess Marines enrolled during the pre-deployment timeframe. During this fourth session we completed assessment of an additional 10 Marines. Our overall post-deployment assessment rate is 31 (49.2%). Post-deployment assessments continued in 2015. All principal analysis is complete and included in publications. The dataset generated will continue to be studied for potential future publications. .

There have been no problems in accomplishing the above tasks.

Key Research Accomplishments:

- All necessary IRB approvals (UCSD, VA, and NHRC) for MRS-II are complete and remain current for continuation of secondary analysis and manuscript writing.
- Data collection for MRS II, Project 1 (Extension study) cohort one is complete
- Pre-deployment data collection for MRS II, Project 2 (Neuro-cognition Study) is complete with an overall retention rate of 74.1%
- All Demonstration projects for MRS II, Project 3, Demonstration project 4 is complete with an overall retention rate of 49.2%
- A draft Marine Resiliency website is currently under review by Headquarters Marine Corps and will be made public after approval (website contains general study information and publications)
- An invention has been submitted (SD2014-043) entitled "Diagnostic and Predictive Metabolite Patterns for Disorders Affecting the Brain and Nervous System" which resulted from findings from the MRS II, Project 3, Demonstration Project 3. This patent addresses use of metabolomics as a biomarker for PTSD.

Reportable Outcomes:

While preliminary outcomes have been reported at meetings, and in published manuscripts, we continue secondary analysis and submit additional manuscripts. We will forward all published future manuscripts to our contacts at NMLC.

We have a number of findings that may be of value to Navy BUMED and HQMC:

1. Based on Project 2 analyses, our findings indicate that longitudinal declines in cognitive performance after deployment were observed in a small subset of subjects endorsing both deployment TBI with LOC≥1 min and prior TBI history. Based on these findings, given that TBI recent exposure and severity were modestly associated with reduced cognitive performance even when controlling for severe TBI (LOC>30 min) and mood and anxiety symptoms. Therefore, TBI history (recent prior TBI or multiple TBI) may be an additional factor to consider when assessing cognitive performance changes associated with TBI.

2. Given the high rates of hearing loss and tinnitus in the military, we sought to examine whether cause, severity, and frequency of traumatic brain injury (TBI) increased risk of post-deployment tinnitus, accounting for co-occurring posttraumatic stress disorder. Pre-deployment TBI increased the likelihood of new-onset post-deployment tinnitus and deployment-related TBIs likewise increased the likelihood of post-deployment tinnitus. The likelihood of new-onset post-deployment tinnitus was highest for those who were blast-exposed, who reported moderate-severe TBI symptoms, and who sustained multiple TBIs across study visits. Posttraumatic stress disorder had no effect on tinnitus outcome.

3. Based on prior cross-sectional evidence for inflammation associated with PTSD, we sought to determine whether inflammation was a risk factor for PTSD. Analysis of our data gives evidence that higher pre-deployment levels of a blood immune marker, c-reactive protein, are a risk factor post-deployment PTSD, accounting for relevant variables such as trauma exposure (Eraly et al., 2014). We sought to confirm evidence of inflammation as an associate of, and risk factor for PTSD through MRS-II gene expression studies. Our gene expression also indicate that there is increased peripheral inflammation associated with PTSD diagnosis, and that inflammation may be a risk factor for PTSD, (Glatt et al., 2013, Tylee et al., 2015, Breen et al., 2015).

4. Our magnetoencephalogram (MEG) study provides further evidence for use of MEG imaging to detect mild traumatic brain injury. The high-resolution MEG source magnitude images obtained by Fast-VESTAL method detected abnormalities at the positive detection rates of 84.5%, 86.1%, and 83.3% for the combined (blast-induced plus with non-blast causes), blast, and non-blast mTBI groups, respectively. We found that prefrontal, posterior parietal, inferior temporal, hippocampus, and cerebella areas were particularly vulnerable to head trauma. Furthermore, MEG slow-wave generation, indicative of injury, in prefrontal areas positively correlated with personality change, trouble concentrating, affective lability, and depression symptoms (Huang et al., 2014). The MEG scan technique was also able to visualize brain abnormalities, different markers of mTBI, associated with PTSD. Those findings, consistent with to those observed using fMRI, were also published (Huang et al, 2014).

5. Based on our metabolomics analyses, PTSD, mild TBI, and well as combined PTSD/mTBI show metabolomic profiles distinct from those of healthy combat-deployed Marine controls. Because the PTSD (without mTBI) metabolomics profiles differ from those of mTBI alone, and those of combined PTSD/mTBI, the total number of subjects was insufficient to provide an internal replication of findings. Thus, these data need to be replicated in a larger cohort.

6. MRS is working collaboratively with other cohorts (e.g. Army STARRS, PRISMO) and with NIH funded consortia to replicate gene expression, epigenetics and genetics findings in PTSD.

Please see Body section for reference to study related abstract presentations and slides cited above.

Conclusions and Recommendations:

1. TBI is a risk factor for development of post-deployment mental health problems, such as depression and PTSD, as well as tinnitus. A small group of multiply exposed Marines may also show cognitive decline.

Thus, policy recommendations might be 1) Employment of methods to document and record intensity of (e.g, blast sensors) of blast exposure(s); 2) Screening for likely consequences (e.g. mental health problems, tinnitus, cognitive decline), and 3) Additional training for Marines about the potential consequences of TBI encouragement to seek early medical assessment for symptoms.

2. Inflammation may be a risk factor for development of PTSD

Thus, policy recommendations might be to reduce likely sources of inflammation prior to and during deployment of military personnel. Such action might include 1) attention to when vaccinations are given; and 2) to the extent possible encourage good sleep hygiene, as sleeplessness increases inflammation. Further research into the mechanisms of the association between inflammation and PTSD would be of value.

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There have been several presentations and pending abstracts on MRS I, MRSII, and related topics.

Study associated presentations:

1. MRS II has provided an informational slide for the Army STARRS/NIH briefing (appendix 1).

2. Dr. Dewleen Baker presented data on the NPY in PTSD, combat control subjects and healthy civilians, as well as prospective plasma NPY concentration trajectory at its relation to combat exposure at the 10th Catecholamine Meeting, Pacific Grove, California on September 10th, 2012 (appendix 2 and 3).

3. Dr. Victoria Risbrough presented an early description and preliminary findings of the fear extinction task at the International Society for Psychoneuroendocrinology Conference in New York, NY on Sept. 12th, 2012 (appendix 4).

4. Dr. Dewleen Baker also contributed to a Panel 1 (Defining optimal biomarkers(s) for the military) of a satellite session of the International Society for Psychoneuroendocrinology Conference entitled "The Use of Biomarkers in the Military: Theory to Practice", led by Colonel Carl Castro and Rachel Yehuda. The session was held in New York, NY on Sept. 14th, 2012

5. Dr. Susan Powell presented data describing the effects of childhood trauma on physiological responses in active duty marines on April 5th, 2013 at the Anxiety and Depression Association of American Annual Conference in La Jolla, CA. and Dr. Risbrough presented a description of the MRS-II study methods and masures on October 31st, 2012 at the BIOS PTSD conference in Los Angeles, CA.

6. Dr. Mingxiong Huang presented data describing "Magnetoencephalography (MEG) source imaging markers for mTBI and PTSD" on August 22, 2013 at the Military Biomarker Conference held in Amsterdam, Netherlands, a satellite conference of the 43rd Annual Meeting of the International Society of Psychoneuroendocrinology, Leiden, Netherlands (appendix 5).

7. Dr. Robert Naviaux presented data on "NextGen Metabolomics for Determining the Predeployment Risk and Postdeployment Diagnosis of PTSD and TBI" on August 22, 2013 at the Military Biomarker conference held in Amsterdam Netherlands, a satellite conference of the 43rd Annual Meeting of the International Society of Psychoneuroendocrinology, Leiden, Netherlands (appendix 6).

8. Dr. Dewleen Baker presented data describing "CRP as a Predictor of PTSD Risk" and "Genetic Risk for PTSD in the Marine Resiliency Study: Interrogation of the Entire Genome" on August 22, 2013 at the 43rd Annual Meeting of the International Society of Psychoneuroendocrinology held in Leiden. Netherlands. Dr. Baker also presented "Military Biomarkers"on August 23th, 2013 at the Satellite Symposium to ISPNE; The Use of Biomarkers in the Military: From PTSD Susceptibility to Disease at the Military Biomarker Conference

held in Amsterdam (appendix 7 and 8). A special issue of Psychoneuroendocrinology is anticipated, entitled: 'Biomarkers in the Military' edited by Drs. Vermetten, Baker and Yehuda. Talks presented (above) will be written up as manuscripts for this issue (appendix 7 and 8).

9. Dr. Dewleen Baker presented data describing "CRP as a Predictor of PTSD Risk" at the 52nd Annual Meeting of the American College of Neuropsycholopharmacology (ACMP) held in Hollywood, FL from December 8-12, 2013 (appendix 7).

10. Dr. Dean T. Acheson presented "Is Deficient Sensorimotor Gating a Pre-Existing Factor in Those That Develop PTSD After Combat Deployment?" at the 52nd Annual Meeting of the American College of Neuropsycholopharmacology (ACMP) held in Hollywood, FL from December 8-12, 2013 (appendix 9).

11. A poster entitled "Prospective Examination of Prepulse Inhibition in OIF/OEF Marines Suggests Reduced Sensorimotor Gating is a Pre-Existing Factor in Those That Develop PTSD After Combat Deployment" (Risbrough VR, Acheson DT, Baker DG, Nievergelt CM, Yurgil KA) was presented at the 52nd Annual Meeting of the American College of Neuropsycholopharmacology (ACMP) held in Hollywood, FL from December 8-12, 2013 (appendix 10).

12. Dr. Baker presented a MRS-II Brief to the 1st MEF Surgeon and staff with an MRS-II update on February 13th, 2014 (appendix 11).

13. A paper entitled "Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in a Sample of Active Duty Marine" (Acheson, DT, Geyer, M, Baker, DG, Neivergelt, CM, Yurgil, KA, Risbrough, VB, & MRS Team) was presented at the 34th annual meeting of the Anxiety Disorders Association of America, Chicago, IL from March 27-March 30th, 2014 (appendix 12).

14. A paper entitled "Genome-Wide Association Study in Combat-Exposed Marines Identifies a Novel Risk Factor for PTSD" (Nievergelt CM, Maihofer AX, Mustapic M, Breen MS, Woelk CH, Whitaker JA, Geyer MA, Risbrough VB, O'Connor DT, Baker DG) was presented at the 69th Annual Scientific Meeting of the Society of Biological Psychiatry, New York, NY on May 8-10th, 2014 (appendix 13).

15. Symposium Entitled New Research Initiatives on Biomarkers in Combat-Related PTSD (Baker, Nievergelt, O'Connor, Risbrough, Maihofer, Cook), with Col. E. Vermetten (Netherlands) and Col. R. Jetly (Canada) Annual Meeting of the International Society for Psychoneuroendocrinology, Montreal, August 22, 2014 (appendix 14).

16. Dr. Mingxiong Huang presented "Developing MEG and DTI markers for PTSD" in a symposium "MEG as a diagnostic tool for post-traumatic stress disorders in military combatants" at the 19th International Conference on Biomagnetism, Biomag2014, Halifax, Canada on August 25, 2014 (appendix 15).

17. Dr. Mingxiong Huang presented "MEG and mild traumatic brain injury" in a symposium "Emerging clinical indications" at the 19th International Conference on Biomagnetism, Biomag2014, Halifax, Canada on August 25, 2014 (appendix 16).

18. A paper entitled "First GWAS in Dopamine Beta Hydroxylase confirms strong cis-acting variants and lends support for its role as an intermediate phenotype in post-traumatic stress disorder" was presented at the 64th Annual Meeting of the American Society of Human Genetics at the San Diego Convention Center (SDCC) in San Diego, California, from October 18-22, 2014 (appendix 17).

19. A poster entitled "Impact of Childhood Maltreatment of Physical Health Related Quality of Life in U.S. Active Duty Servicemen and Veterans" (Aversa L, Lemmer J, Nunnik S, McLay R, Baker DG) was presented at the 30th Annual Meeting of the International Society for Traumatic Stress Studies held in Miami, Florida from November 6-8, 2014 (appendix 18).

20. A poster entitled "Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in a Sample of Active Duty Marines" (Acheson DT, Geyer M, Baker DG, Nievergelt CM,

Yurgil KA, Risbrough VR) was presented at the 52nd Annual Meeting of the American College of Neuropsycholopharmacology (ACMP) held in Phoenix, Arizona from December 7-11, 2014 (appendix 19).

21. A poster entitled "Identifying Extinction Learning Trajectories and their Association with Psychiatric Symptoms in a Sample of Active Duty Marines." (Acheson DT, Baker DG, Geyer M, Risbrough VR) was presented at the Annual Meeting of the Anxiety and Depression Association of America (ADAA) held in Miami, Florida on March 31-April 3, 2015 (appendix 20).

22. A poster entitled "Acoustic Startle Threshold: Predictor of Psychiatric Symptoms Pre- and Post-deployment" (Glenn DE, Acheson DT, Nievergelt CM, Baker DG, Risbrough VR) was presented at the Annual Meeting of the Anxiety and Depression Association of America (ADAA) held in Miami, Florida on March 31-April 3, 2015 (appendix 21).

23. Dr. Nievergelt presented "Extending Genome-Wide Associations Studies to Identify Risk Factors for PTSD in combat-exposed Marines." at the Annual Meeting of the Anxiety and Depression Association of America (ADAA) held in Miami, Florida on March 31-April 3, 2015 (appendix 22).

24. A poster entitled "Acoustic Startle Threshold: Predictor of Psychiatric Symptoms Pre- and Post-deployment" (Glenn DE, Acheson DT, Nievergelt CM, Baker DG, Risbrough VR) was presented at the Lewis L. Judd Young Investigators Symposium at UCSD on April 13, 2015 (appendix 21).

25. Dr. Nievergelt presented "Genomic Predictors of Combat Stress Vulnerability in U.S. Marines: Genome-wide Association Studies across Multiple Ancestries Identify Novel Risk Factors for PTSD" at the 2015 Society of Biological Psychiatry Annual Meeting on May 14-16, 2015 (appendix 23).

26. A poster entitled "MEG Imaging Markers for Mild TBI and PTSD." (Huang MX, Baker DG) was presented at the Military Health System Research symposium was held from Aug 17-20 2015 at the Marriott Harbor Beach Resort, Ft. Lauderdale, FL (appendix 24).

27. Dr. Baker presented "Autonomic Nervous System and Immune Markers of PTSD Risk and Resilience at the 2015 Military Health System Research symposium on Aug 19, 2015 at the Marriott Harbor Beach Resort, Ft. Lauderdale, FL (appendix 25).

28. A poster entitled "MEG Imaging Markers for Mild TBI and PTSD." (Huang MX, Baker DG) was presented at the State-of-the-Art (SOTA) meeting on Traumatic Brain Injury (TBI) in Veterans being sponsored by VA's Office of Research and Development on August 24-25, 2015 (appendix 24).

29. Dr. Baker presented "Seeking Risk and Resilience Factors for PTSD: The MarineResiliency Study" at the Clinical, Translational Research Institute, University of California, San Diego on March 25, 2016 (appendix 26).

Please see appendices for slides and abstracts.

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Conditioned fear and extinction learning performance and its association with psychiatric symptoms in active duty Marines

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Safety signal;
Startle

Summary
Background: Posttraumatic Stress Disorder (PTSD) is a major public health concern, especially given the recent wars in Iraq and Afghanistan. Nevertheless, despite a sharp increase in the incidence of psychiatric disorders in returning veterans, empirically based prevention strategies are still lacking. To develop effective prevention and treatment strategies, it is necessary to understand the underlying biological mechanisms contributing to PTSD and other trauma related symptoms.
Methods: The "Marine Resiliency Study II" (MRS-II; October 2011–October 2013) Neurocognition project is an investigation of neurocognitive performance in Marines about to be deployed to Afghanistan. As part of this investigation, 1195 Marines and Navy corpsmen underwent a fear conditioning and extinction paradigm and psychiatric symptom assessment prior to deployment. The current study assesses (1) the effectiveness of the fear potentiated startle paradigm in producing fear learning and extinction and (2) the association of performance in the paradigm with baseline psychiatric symptom classes (healthy: $n=923$, PTSD symptoms: $n=42$, anxiety symptoms: $n=37$, and depression symptoms: $n=12$).
Results: Results suggest that the task was effective in producing differential fear learning and fear extinction in this cohort. Further, distinct patterns emerged differentiating the PTSD and anxiety symptom classes from both healthy and depression classes. During fear acquisition, the PTSD symptom group was the only group to show deficient discrimination between the

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conditioned stimulus (CS+) and safety cue (CS−), exhibiting larger startle responses during the safety cue compared to the healthy group. During extinction learning, the PTSD symptom group showed significantly less reduction in their CS+ responding over time compared to the healthy group, as well as reduced extinction of self-reported anxiety to the CS+ by the end of the extinction session. Conversely, the anxiety symptom group showed normal safety signal discrimination and extinction of conditioned fear, but exhibited increased baseline startle reactivity and potentiated startle to CS+, as well as higher self-reported anxiety to both cues. The depression symptom group showed similar physiological and self-report measures as the healthy group.

Discussion: These data are consistent with the idea that safety signal discrimination is a relatively specific marker of PTSD symptoms compared to general anxiety and depression symptoms. Further research is needed to determine if deficits in fear inhibition vs. exaggerated fear responding are separate biological "domains" across anxiety disorders that may predict differential biological mechanisms and possibly treatment needs. Future longitudinal analyses will examine whether poor learning of safety signals provides a marker of vulnerability to develop PTSD or is specific to symptom state.

1. Introduction

Posttraumatic Stress Disorder (PTSD) is a major public health concern among current and former military members, including those who have recently experienced combat in Iraq and Afghanistan (Baker et al., 2012). For instance, while most service members remain resilient following deployment, the incidence of psychiatric disorders among active-duty service members has increased by 62% since these wars began in 2001. Specifically, there has been an increase of 656% for PTSD and 226% for anxiety disorders. In addition, the cost to the Department of Defense (DoD) for treating these service members doubled between 2007 and 2012 (Blakeley and Jansen, 2013 Congressional Research Service Report). The Department of Veterans Affairs (VA) and society at large will continue to bear the cost of treating service members with chronic psychiatric issues long after these individuals are discharged from the military. According to a recent report by the Institute of Medicine, DoD prevention efforts are hampered by an insufficient empirical base (National Research Council, 2014). Identifying the underlying biological mechanisms of PTSD from other stress-related disorders is a key step in developing an evidence base on which to design more effective prevention and treatment efforts.

The "Marine Resiliency Study II" (MRS-II; October 2011–October 2013) Neurocognition project is an investigation of neurocognitive performance in Marines about to be deployed to Afghanistan. Similar to the original MRS (Baker et al., 2012), Marines were assessed in a 3.5 h test battery in which clinical assessment, self-report, and biological assays are combined with comprehensive neurocognitive assessments once before deployment and then again 3–6 months after deployment. The purpose of MRS-II is to discriminate between biological markers that predict risk/resiliency for development of combat-stress related disorders and markers associated specifically with symptom state. Here we focus on one aspect of these assessments, measurement of fear conditioning and extinction learning and its association with psychiatric symptom groups prior to deployment.

Increased responses to conditioned fear cues and reduced ability to inhibit these responses are well-known features of PTSD in civilian and combat-veteran populations (for review see VanElzakker et al., 2013). Reduced ability to inhibit fear has recently been suggested to be a potential "biomarker" specific to PTSD, with PTSD subjects exhibiting poor learning of safety signals (cues that predict absence of threat) compared to depressed subjects (Jovanovic and Norrholm, 2011; Jovanovic et al., 2009, 2010). Studies in high trait anxious participants or other anxiety disorders are inconsistent, showing either normal or reduced fear inhibition as measured by safety signal learning (Kindt and Soeter, 2014; Gazendam et al., 2013; Lissek et al., 2009). Reduced inhibition in PTSD patients is thought to reflect disruption of frontal cortical and hippocampal circuits to inhibit amygdala activation and concomitant fear responses (Admon et al., 2013; Acheson et al., 2012a,b). However, increased fear responding to conditioned cues, aversive contexts, or overgeneralization of fear responses are shown across multiple anxiety disorders and thus may reflect biological processes that are shared across disorders (McTeague and Lang, 2012; Lissek et al., 2013; Grillon et al., 1998). Results are less clear however for depression, with reports of lower, normal, and higher aversive responding or fear conditioning (McTeague and Lang, 2012; Grillon et al., 2013; Robinson et al., 2012; Jovanovic et al., 2010) depending on the type of conditioned cues and aversive stimuli. Heightened fear responding may be due to increased amygdala, extended amygdala, and/or dorsal anterior cingulate activity in these disorders (Admon et al., 2013; Grillon, 2008). Understanding the differential patterns of fear conditioning and inhibition between symptom types will help identify specific endophenotypes for further biological interrogation across stress-related disorders (Cuthbert and Kozak, 2013; McTeague and Lang, 2012; Admon et al., 2013). Given that MRS-II is a longitudinal study, we will ultimately be able to determine in future analyses if these putatively differential phenotypes are vulnerability factors or related specifically to symptom state after trauma.

To test the hypothesis that PTSD, depression, and general anxiety symptoms may reflect distinct biological mechanisms and subsequent differential patterns of fear conditioning and inhibition abnormalities, we used a cross-sectional design to directly compare fear conditioning and

extinction across participants endorsing symptoms of general anxiety, depression, and PTSD at pre-deployment. We used the fear potentiated startle (FPS) paradigm established by Norrholm et al. (2006), as this paradigm is sensitive to both the reduced fear inhibition (i.e., safety signal learning and extinction) and increased fear conditioning described in PTSD patients (Norrholm et al., 2011). This protocol uses an aversive air-puff as the unconditioned aversive stimulus. Though other fear conditioning paradigms have used aversive electrical shock as the unconditioned stimulus (i.e., Milad et al., 2007), we chose to use air puff for a number of reasons. One, use of an air puff increased the feasibility of testing such a large active duty population in a time-limited manner as it does not require initial "customization" of shock stimuli. Lack of required customization reduced setup time as well as technical difficulty. Two, we anticipated that shock stimuli would be less acceptable to study participants and to local and military institutional review boards given the special population status of active duty military. Third, this protocol uses startle reactivity as the operational measure of conditioned fear, a cross species measure of fear conditioning for translational applications in animal models, and which may be more sensitive to "automatic" or implicit fear learning compared to other measures such as skin conductance (Sevenster et al., 2014; Glover et al., 2011).

2. Methods

2.1. Participants

1195 infantry Marines and Navy Corpsmen enrolled in a longitudinal study of the health effects of deployment to Afghanistan and completed the pre-deployment assessment. Data was collected on two separate infantry battalions, identified with the assistance of Marine Corps leadership, 1–2 mo prior to deployment. The first battalion was deployed from March 2012 to October 2012, and the second battalion from September 2012 to April 2013. At the time of this collection period all Marine infantry were male, thus females did not participate. All data collection occurred on a single day, with the entire testing battery (of which only a portion is being presented here) was completed over the course of approximately 4 h. This study was approved by the institutional review boards of the University of California San Diego, VA San Diego Research Service, and the Naval Health Research Center. Written informed consent was obtained from all participants.

2.2. Fear conditioning and extinction procedure

Apparatus: Startle pulses (108 dB, 40 ms) were delivered using a San Diego Instruments (SDI, San Diego, CA, USA) SR-HLAB Electromyography (EMG) system. Sound levels were measured using continuous tones calibrated with a Quest Sound Level Meter on the A scale, coupled to the headphones with an artificial ear. The air puff was set at 250 psi and delivered via a plastic tube positioned 2.5 cm from the center of the throat. Air-puff onset was controlled by a solenoid system triggered by the same Acer laptop computer that controlled the startle stimuli. Conditioned stimuli were presented via E-Prime software (Psychology Software Tools, Inc., Sharpsburg, PA, USA) run on a Dell desktop computer with a 48 cm monitor positioned directly in front of the participant. Presentation of the stimuli by the E-Prime software was triggered by signals from the EMG system to control synchronization of conditioned, startle, and air-puff stimuli.

Eyeblink EMG responses were recorded via Ag/Ag 3 M Red Dot electrodes placed at the *orbicularis oculi* muscles at the left eye connected to the SDI SR-HLAB EMG system and Acer laptop computer (Acheson et al., 2013, 2012a,b). A reference electrode was placed at the mastoid bone behind the left ear. Before electrode placement, skin was cleaned with an alcohol swab and gently exfoliated with 3 M electrode prep tape. All electrode resistances were <10 kΩ. EMG data were recorded at a sampling rate of 1 kHz, amplified (0.5 mV electrode input was amplified to 2500 mV signal output), band-pass filtered (100–1000 Hz), rectified, and then smoothed with a 5-point rolling average. Expectancy responses were recorded on a trial-by-trial basis via the participant's responses on a key pad linked to E-Prime software. Additional self-report responses were recorded at the end of each experimental phase via the same keypad.

Eyeblink data were scored via SR-HLAB EMG Utilities software as previously described (Acheson et al., 2012a,b). In brief, eyeblink responses were examined on a trial by trial basis at a window starting 100 ms before the startle pulse and ending 200 ms after the pulse. Only responses that peaked within 100 ms of pulse onset were scored as a startle response. Trials in which excessive baseline noise or artifact obscured the startle response were removed (2.1% of trials) and replaced with an imputed value based on the average of the immediately preceding and following trials.

Fear conditioning and extinction task: The fear conditioning and extinction protocol consisted of two discrete testing sessions or "phases": acquisition and extinction. Before the acquisition phase the participants were instructed that one of the colored symbols predicted when the air puff would appear. Each phase began with 6 startle pulses presented in the absence of any other stimuli to stabilize startle responding. The acquisition phase consisted of eight 6-s presentations of the conditioned stimulus (CS+; either a blue or yellow circle or square, balanced across subjects) that was paired with the air puff in 75% contingency, eight 6-s presentations of a non-reinforced conditioned stimulus (CS-; also either a blue or yellow circle or square) that was never paired with the air puff, and 8 presentations of the startle stimulus in the absence of any stimuli (noise alone or "NA" trial) which served as a measure of baseline startle across the phase. The CS+ and air puff co-terminated on reinforced trials. Startle pulses were presented approximately 4 s following CS+ or CS− onset. The stimuli serving as CS+ and CS− (blue or yellow circles or squares) were randomly assigned across participants. Contingency awareness was measured using a numbered keypad to report at each CS+ and CS− trial whether or not they expected to receive the air puff. Participants responded with a "1" if they expected the air puff, "2" if they were unsure, and "3" if they did not expect the air puff. After the acquisition phase, contingency awareness was again assessed via a questionnaire

asking participants which stimulus predicted the shock. Self-reported anxiety during the cues was also measured at this time, as was the subjective aversiveness of the air-puff stimuli.

After completing the acquisition phase, participants were asked to sit quietly for 5 min before beginning the extinction phase. Before the extinction phase began, the subjects were told to "remember what they learned" in the previous session. The extinction phase consisted of 16 presentations of each stimulus type (CS+, CS−, and NA). No air puffs were presented during this phase. Presentations of startle pulses were delivered and ratings of air-puff expectancy were collected in the same fashion as in the acquisition phase. After this phase, participants again rated their level of anxiety during the cues. After these ratings were made, participants were disconnected from the apparatus and went on to other assessment stations (see Baker et al. (2012) for full details of Marine Resiliency Study assessment battery).

2.3. Assessment of psychiatric symptoms

Posttraumatic Stress Disorder: Posttraumatic stress symptoms were assessed using a structured diagnostic interview, the Clinician Administered PTSD Scale (CAPS; Blake et al., 1995). CAPS total scores can range from 0 to 136 and can be used as a measure of PTS symptom severity. PTSD symptom group membership was defined using the partial PTSD criteria articulated by Stein et al. (1997). Partial PTSD criteria were chosen due to the relative psychological health of an active duty Marine cohort. Criteria for assignment to the PTSD symptom group were the presence of at least 1 B symptom, 2 C symptoms, and 2 D symptoms, with minimum frequency ratings of 1 and minimum intensity ratings of 2. Inter-rater reliability in MRS was high for both the CAPS total score (intraclass correlation coefficient = .99) and for PTSD diagnosis (kappa = .714). All interviews were conducted by study personnel who were trained, certified and supervised by a licensed psychiatrist (D.G.B.; Baker et al., 2012).

Anxiety: Assignment to the anxiety symptoms group was defined as scoring in the Moderate to Severe range (>15) on the Beck Anxiety Inventory (BAI; Beck and Steer, 1993). The BAI is a reliable measure of general anxiety symptoms present within the past week, and discriminates between anxiety vs. depressive symptoms fairly well (Clark et al., 1994).

Depression: Assignment to the depression symptoms group was defined as scoring in the Moderate to Severe range (>19) on the Beck Depression Inventory 2 (BDI-2; Beck et al., 1996). The BDI-2 measures the presence of depressive symptoms within the past 2 weeks.

Trauma history: The Life Events Checklist (LEC; Gray et al., 2004) was used to assess previous trauma history. The LEC evaluates the participant's lifetime experience of a wide range of traumatic events, including civilian traumas and combat or war-zone exposure, and further assesses whether the event directly happened to the individual, the individual witnessed the event happening to others, or whether the event was learned about second-hand. The LEC score reported here was calculated by summing all of the items scored as "happened to me" and/or "witnessed it".

2.4. Data analysis

Final sample: Of the original 1195 Marines and Corpsmen who underwent the fear conditioning and extinction protocol, data on 21 were rendered unusable due to technical difficulties during testing. An additional 125 (10.6% of the remaining sample) were excluded from the analysis because they failed to show a CS+ response greater than baseline during the last half of the acquisition phase. This failure to potentiate above baseline suggested that the air puff was ineffective in inducing fear in these subjects that would be sufficient to support learning in these participants. Further, 35 subjects met our cutoffs for more than one symptom group and were excluded from the analysis. This approach was taken to enable comparison of relatively "pure" symptom classes on fear conditioning and extinction phenotypes. See supplemental materials Table S1 for demographic data on these excluded subjects. The remaining 1014 subjects were included in all analyses.

Startle: Startle data for the acquisition and extinction phases were analyzed as previously described in Acheson et al. (2013) by averaging responses to each stimulus type into blocks of two trials. Within each block, the NA averages were subtracted from the CS+ and CS− averages to adjust for changes in baseline startle across the session. Thus, each CS+ and CS− block represented startle above baseline for that block (e.g., (CS+) − (NA)). Thus there were 4 blocks for the CS+ and CS− during the acquisition phase, and 8 blocks for the CS+ and CS− for the extinction phase.

To compare acquisition across symptom groups, the analysis was simplified by averaging the last two blocks of the session across both CS types to create a measure of responding over the last half of the acquisition phase. To assess function of the task, acquisition phase data were initially analyzed within the healthy group only using a repeated-measures ANOVA to assess differences in response to each CS type. To assess differences by symptom group, a 2 (CS type) × 4 (symptom group) mixed ANOVA was conducted on the entire sample. Significant interactions were followed up with alpha-adjusted post hoc tests to assess Cue response differences within each symptom group. To assess symptom group differences in baseline startle, a one-way ANOVA, with appropriate post hoc tests, was conducted on the average NA trial response across the last half of the extinction phase.

Extinction phase data were analyzed by computing a measure of "% conditioned fear". This score is similar to the "extinction retention index" originated by Milad et al. (2007, 2008) in their studies of fear extinction memory recall, which use a normalization approach to reduce confounds of differences in fear conditioning on measurement of extinction. For each subject, the maximal CS+ response during the acquisition phase is identified. A % conditioned fear is then calculated for each of the 8 extinction blocks using the following equation: 100*(CS+ response on extinction block/maximum response across acquisition blocks). For simplicity of presentation and analysis, these scores were further averaged into 4 extinction blocks consisting of 4 trials each. The first block, Early Extinction, consisted of the first 4 trials of the phase, Mid Extinction 1 trials 5–8, Mid Extinction 2 trials 9–12, and Late Extinction trial 13–16. To assess function of the task,

extinction phase data were initially analyzed within the healthy group only using a repeated-measures ANOVA to assess decrease in responding across the phase. To assess differences by symptom group, a 4 (symptom group) × 4 (Extinction Block) mixed ANOVA was conducted on the entire sample. To assess symptom group differences in baseline startle response during the extinction phase, a 4 (symptom group) × 4 (Extinction Block) mixed ANOVA, with appropriate post hoc tests, was conducted on the NA responses averaged into blocks analogous to those above.

Expectancy and self-report: Expectancy responses were re-coded as: expect air puff = 1, unsure = 0, do not expect air puff = −1. Expectancy responses over the last half of the acquisition phase (4 trials/stimulus type) were averaged together as with the startle data. ANOVAs were applied to assess both task effectiveness and differences by symptom group in the same manner as with the startle responses.

Expectancy responses during the extinction phase were analyzed by trial, including the last 4 trials of the acquisition phase (20 total trials). Task effectiveness was assessed using a repeated-measures ANOVA on the healthy group only. A 4 (symptom group) × 20 (trial) mixed ANOVA was used to assess differences by symptom group across the entire sample.

To assess task effectiveness on self-reported anxiety, CS type differences on post-phase questionnaires were analyzed using repeated measures ANOVA on the healthy group alone. A 2 (CS type) × 4 (symptom group) mixed ANOVA was used to assess differences across symptom groups. Task effectiveness in assessing change across phase in self-reported anxiety was assessed using a repeated-measures ANOVA in the healthy group only. Differences across phase by symptom group were assessed with 4 (symptom group) × 2 (phase) mixed ANOVA on the entire sample. In all analyses,

Table 1 Demographics and symptom measures.

	Symptom group			
	Healthy	PTSD	Anxiety	Depression
N	923	42	37	12
Age (SD)	22.23 (2.81)	22.63 (4.08)	22.4 (3.27)	21.38 (2.33)
Months in the military (SD)	31.29 (26.18)	39.5 (43.89)	32.7 (28.74)	31 (29.64)
Education				
<H.S.	3.3%	2.4%	2.7%	8.3%
H.S.	69.3%	76.2%	73%	91.7%
Some college	25%	21.4%	21.6%	0%
B.A.	2.4%	0%	2.7%	0%
Post-graduate	0%	0%	0%	0%
Rank				
Junior enlisted	71.3%	76.2%	78.4%	91.7%
NCO	27.5%	23.8%	18.9%	8.3%
Officer	1.2%	0%	2.7%	0%
Race				
White	87.4%	85.7%	83.3%	83.3%
African-American	3.7%	0%	0%	0%
Other	8.9%	14.3%	16.2%	16.6%
Ethnicity				
Not Hispanic or Latino	75.8%	64.3%	67.5%	75%
Hispanic or Latino	24.2%	35.7%	32.4%	25%
Marital status				
Single, never married	68.5%	69%	75.7%	75%
Married	29.3%	28.6%	21.6%	25%
Divorced	1.4%	2.4%	0%	0%
Separated	0.9%	0%	2.7%	0%
Pathology measures (SD)				
CAPS total score	9.66[a] (9.34)	43.74 (11.29)	17.95[a] (10.91)	27.83[a] (12.06)
BAI total score	2.87[a] (4.03)	4.4[a] (5.54)	20.41 (5.45)	6.67[a] (4.92)
BDI-2 total score	3.89[a] (4.19)	9.86[a] (5.43)	9.65[a] (5.44)	24.17 (3.33)
LEC score	4.16 (2.80)	5.93[b] (3.60)	5.54[b] (3.12)	5.92[b] (2.27)

[a] $p < .05$ for comparisons vs. category reference group (i.e., PTSD group reference for CAPS score comparisons).
[b] $p < .05$ vs. healthy.

significant interactions were followed up with two-tailed Tukey post hoc tests.

3. Results

3.1. Demographics

Sample demographics are displayed in Table 1. There were no differences across symptom groups on any demographic variable. Differences between symptom groups did emerge on the LEC [$F(3,1010) = 9.03$, $p < .0001$, partial $\eta^2 = .03$], such that all symptom groups reported more trauma experience relative to healthy controls ($ps < .04$). However, the symptom groups did not differ from one another. Two subjects were taking psychiatric medication for reasons other than smoking cessation or sleep (1 in the PTSD symptom group and 1 in the anxiety symptom group). Both of those subjects reported taking fluoxetine at unknown dosages. As expected from our selection criteria, the symptom groups had significantly higher scores on their respective assessment measures relative to the other groups (Table 1; omnibus tests $F(3,1010) > 129.55$, $ps < .0001$; $ps < .05$ for comparisons vs. reference group). All symptom groups had higher levels of PTSD, anxiety and depression symptoms compared to controls healthy controls ($ps < .05$).

3.2. Overall task effectiveness

3.2.1. Acquisition

Startle: As expected, startle responses during the Acquisition phase showed a significant effect of Cue type, with the CS+ response being elevated relative to the CS−, indicating successful differential fear conditioning [Fig. 1A, $F(1,918) = 475.14$, $p < .0001$, partial $\eta^2 = .34$].

Expectancy and self-report: For expectancy ratings, participants correctly identified the CS+ as predictive of the shock [Fig. 2A; $F(1,913) = 3916.39$, $p < .0001$, partial $\eta^2 = .811$]. On a 1 (expect air puff) to −1 (do not expect air puff) scale, participants averaged a 0.59 rating for the CS+ and a −0.78 rating for the CS−.

On the post-phase questionnaire, 88.9% of participants correctly identified the CS+ as predictive of the air puff. 6.7% of participants were not sure which CS predicted the air puff, and 3.1% misidentified the CS− as predictive of the air puff. Overall, participants assigned the air puff an average aversiveness rating of 2.31 out of 5 (SD = 1.02). Participants rated higher levels of subjective anxiety in the presence of the CS+ relative to the CS-, again indicative of differential fear conditioning [Fig. 1C; $F(1,911) = 1298.43$, $p < .0001$, partial $\eta^2 = .588$].

3.2.2. Extinction

Startle: As expected, percentage of conditioned fear (normalized to the fear levels displayed in the acquisition phase) decreased significantly across the phase, demonstrating successful fear extinction [Fig. 2A; $F(3,2751) = 182.87$, $p < .0001$, partial $\eta^2 = .166$].

Expectancy and self-report: Expectancy ratings to the CS+ decreased significantly across the late acquisition and

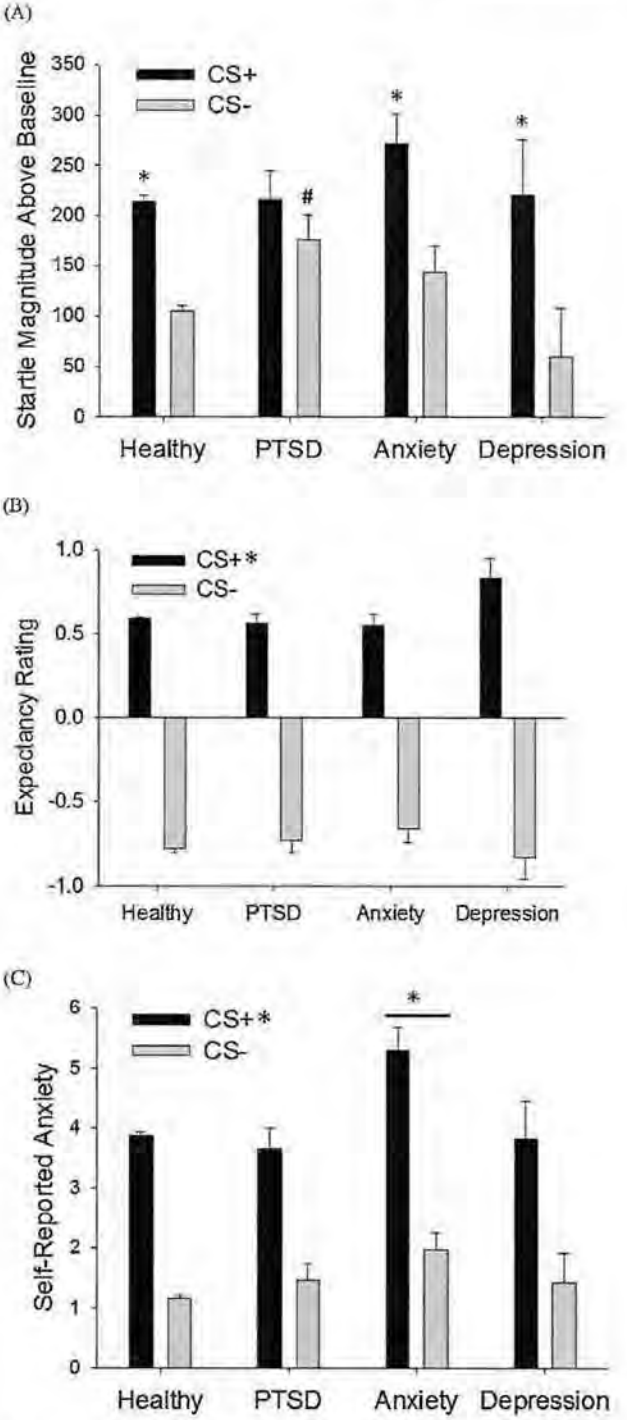

Figure 1 (A) Potentiated startle magnitudes across the last half of the acquisition phase by symptom group. $^{*}p < .05$ for CS+ vs. CS− comparisons. $^{\#}p < .05$ for PTSD symptoms vs. healthy comparison. (B) Expectancy ratings across the last half of the acquisition phase by symptom groups. $^{*}p < .05$ for the CS+ vs. CS− main effect. (C) Self-reported anxiety by symptom groups following the acquisition phase. $^{*}p < .05$ for CS+ vs. CS− main effect and anxiety symptoms vs. healthy comparison.

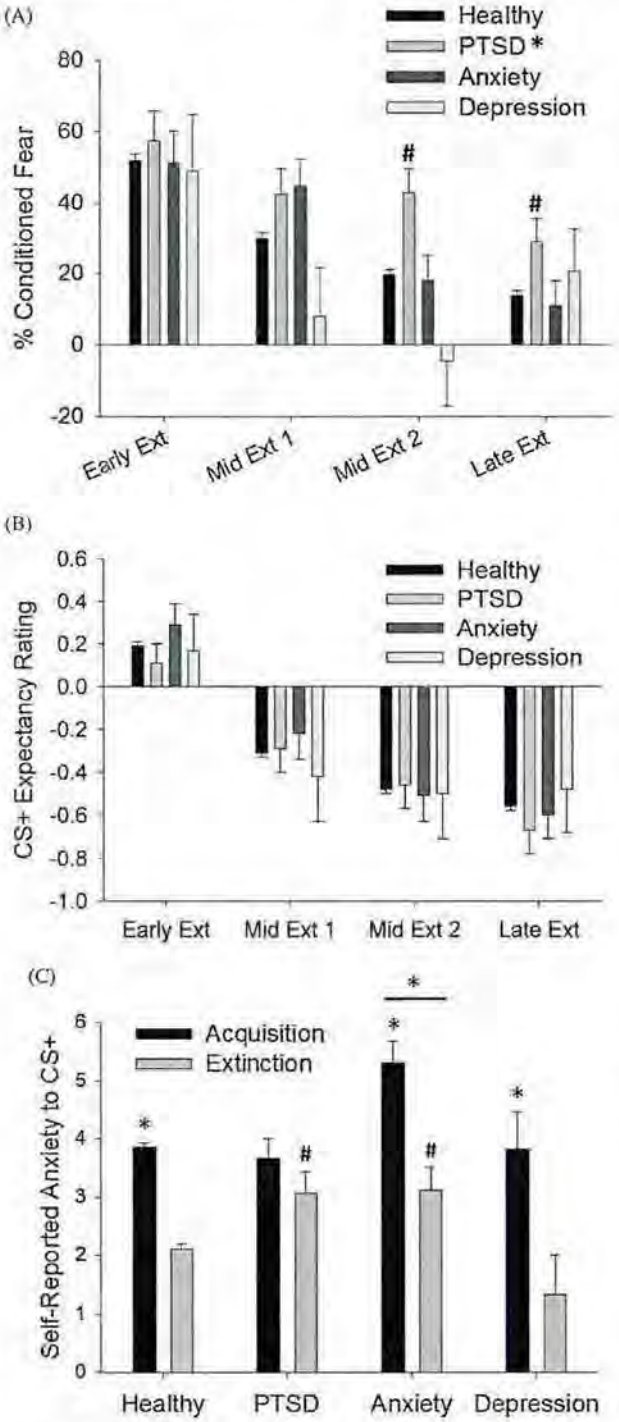

Figure 2 (A) % acquisition response retained across the extinction phase by symptom group. *$p < .05$ for PTSD symptoms vs. healthy comparison. #$p < .05$ for exploratory comparisons vs. healthy controls. (B) CS+ expectancy ratings across the entire extinction phase. Ratings have been combined into 4-trial blocks for clarity. (C) Self-reported anxiety following the acquisition and extinction phases by symptom group. *$p < .05$ for comparisons across phase and for the anxiety symptoms vs. healthy comparison. #$p < .05$ for PTSD and anxiety symptoms vs. healthy comparisons within the extinction phase.

extinction phases [Fig. 2B; $F(19,16682) = 573.56$, $p < .0001$, partial $\eta^2 = .395$]. From the acquisition to extinction phases, post-phase ratings of anxiety to the CS+ decreased significantly [Fig. 3B; $F(1,902) = 529.15$, $p < .0001$, partial $\eta^2 = .37$].

3.3. Comparison of task performance between psychiatric symptom groups

3.3.1. Acquisition

Baseline startle: There was a significant difference between symptom groups in average baseline startle during the last half of the acquisition phase [$F(3,1010) = 3.05$, $p < .03$, partial $\eta^2 = .009$], such that the anxiety symptom group had a higher magnitude of startle relative to healthy controls ($p < .009$). No other symptom group differed from healthy controls.

Startle potentiation: When participants meeting criteria for inclusion in a symptom group were examined, a significant symptom group × Cue type interaction emerged [Fig. 1A; $F(3,1005) = 3.4$, $p < .02$, partial $\eta^2 = .01$]. Post hoc tests revealed that responding to the CS+ was significantly higher than responses to the CS− for the healthy, anxious, and depressed symptom groups ($ps < .001$), but not for the PTSD symptom group ($p < .09$) suggesting reduced differential fear conditioning in the PTSD symptom group. This deficit in differential conditioning was driven by higher CS− responses in the PTSD symptom group relative to the healthy group ($p < .004$). In contrast, the anxiety symptom group exhibited a trend for increased CS+ responding ($p < 0.06$) and no significant differences in CS− responses compared to healthy controls. Maximum CS+ responding was also calculated across the groups, and the anxiety symptom group showed significantly larger maximum CS+ responses compared to the healthy group [supplemental Fig. 1; $F(3,1010) = 2.73$, $p < .05$, partial $\eta^2 = .008$; anxiety symptoms vs. healthy $p < .02$]

Expectancy and self-report: For expectancy ratings, there was no symptom group × Cue type interaction [Fig. 2A; $F(3,1000) = 1.62$, ns], nor was there an overall effect of symptom group [$F(3,1000) < 1.0$, ns]. For self-reported anxiety, there was a significant effect of symptom group [Fig. 3A; $F(3,997) = 5.78$, $p < .001$, partial $\eta^2 = .017$] with anxious subjects reporting higher levels of anxiety in response to both cues ($p < .001$). There was no symptom group × Cue type interaction [$F(3,997) = 1.65$, ns].

3.3.2. Extinction

Baseline startle: There was a trend toward differential responding between symptom groups across the extinction phase [$F(3,1010) = 2.09$, $p < .1$, partial $\eta^2 = .006$], again with the anxiety symptom group trending toward higher response relative to healthy controls ($p < .1$).

Startle potentiation: A significant main effect of symptom group was apparent on %conditioned fear during the extinction phase [$F(3,1005) = 3.05$, $p < .03$, partial $\eta^2 = .009$], such that the PTSD symptom group maintained a higher level of conditioned fear across the entire session compared to the healthy controls ($p < .006$). There was also a trend for a block × symptom group interaction [Fig. 2A; $F(9,3015) = 1.66$, $p < .1$, partial $\eta^2 = .005$]. Exploratory post hoc analyses at each block showed that the PTSD symptom

group maintained a higher level of conditioned fear relative to healthy controls at both the Mid Extinction 2 and Late Extinction blocks (*ps* < .05). The anxiety symptom group showed a trend toward higher responding relative to controls during Mid Extinction 1 ($p < .07$), however this trend was not apparent at the later extinction blocks. The depression symptom group did not differ from healthy controls.

Expectancy and self-report: Expectancy ratings to the CS+ did not vary by symptom group across the phase [Fig. 2B; $F(45,14505) = 1.33$, ns], nor was there a main effect of symptom group [$F(3,967) < 1.0$, ns]. For self-reported anxiety, there were significant differences in change across phases by symptom group [Fig. 3B; $F(3,988) = 4.24$, $p < .01$, partial $\eta^2 = .013$], such that all groups showed significant reductions across phase (*ps* < .05) with the exception of the PTSD symptom group. The PTSD and anxiety symptom groups had higher responses to the CS+ during the extinction phase relative to the healthy group (*ps* < .02). In addition, there was a significant main effect of symptom group, with the anxiety symptom group showing higher ratings overall relative to the healthy group [$F(3,988) = 5.12$, $p < .002$, partial $\eta^2 = .015$].

4. Discussion

As expected, the conditioning paradigm was effective in producing conditioned fear learning and subsequent extinction learning in our active-duty Marine and Navy volunteers. Psychiatrically healthy participants acquired differential fear-potentiated startle and self-reported anxiety responses to the CS+ vs. the CS− and showed contingency awareness (expectancy ratings). Across the extinction phase, when the air puff was absent, responses to the CS+ decreased in terms of both potentiated startle and self-reported anxiety. Expectancy ratings showed intact contingency learning across extinction as well. Successful learning in this paradigm enables comparisons to be made in the learning patterns among the various psychiatric symptom groups.

Differential patterns of learning performance emerged between psychiatric symptom groups. The PTSD symptom group was unique in failing to show a differential potentiated startle response to CS+ and CS− at the end of fear acquisition. This failure was due to PTSD symptom group subjects maintaining a relatively high startle response to the CS−. The observation of high startle responses to the CS− is in line with existing research showing that individuals with PTSD have difficulty learning to inhibit startle responses in the presence of a safety signal (Jovanovic et al., 2009, 2010). Though not explicitly termed "safety signal" in the current paradigm, presentation of the CS− effectively signals the absence of the air puff, or safety. Interestingly, the participants in the PTSD symptom group showed intact contingency awareness in the expectancy ratings, as well as intact discrimination learning as assessed by self-reported anxiety. These findings suggest a "disconnect" between the participant's explicit experience and automatic physiological responses to the safety cue (i.e., potentiated startle).

Across the extinction phase, the PTSD symptom group maintained potentiated startle to the CS+ overall relative to the healthy group. The finding that conditioned fear responses were maintained throughout extinction supports existing research suggesting a disruption in fear extinction learning and recall in PTSD symptom group subjects relative to healthy controls (Norrholm et al., 2011; Milad et al., 2008; Wessa and Flor, 2007; Orr et al., 2000; Peri et al., 2000). This greater maintenance of conditioned fear was also apparent in the self-report of anxiety in response to the CS+, which remained relatively unchanged in the PTSD group after extinction training, unlike the other groups. Again, the PTSD symptom group showed normal explicit learning that the CS+ no longer predicted the US (as evidenced by the expectancy ratings across the extinction session), further supporting a disconnect between explicit contingency awareness and fear expression. Thus the current findings of deficient inhibition of potentiated startle to a safety cue and reduced extinction of physiological and emotional fear responses in the presence of intact contingency awareness supports the theory that PTSD is characterized by a failure to inhibit automatic, physiological fear responses. This failure of inhibition is observed even though the subject is explicitly aware of a lack of threat or danger.

The anxiety symptom group showed significantly higher baseline startle responding and higher CS+ potentiation compared to the healthy group. This group also reported significantly higher anxiety to both CS+ and CS− after acquisition relative to the healthy group. The finding that CS+/− discrimination is normal in participants with high generalized anxiety symptoms is in line with other report that high trait anxiety participants exhibit normal CS+/CS− discrimination (Kindt and Soeter, 2014; Gazendam et al., 2013). The present findings of higher self-reported anxiety to the conditioned cues are also in line with past reports using a similar protocol (Gazendam et al., 2013). During extinction training, the anxiety symptom group successfully extinguished both potentiated startle and US expectancy to the CS+. They also successfully extinguished self-reported anxiety to the CS+, however overall responding remained high compared to the other groups. Taken together, this pattern of results is suggestive of greater explicit anxiety responses during aversive anticipation in this group while fear inhibition and discrimination processes are relatively normal.

The depression symptom group showed response patterns in all measures that were indistinguishable from healthy controls. The normal fear inhibition and potentiated startle in the depression group as assessed by safety signal learning and extinction is in line with previous studies (Jovanovic et al., 2010, 2012). The present results differ however from a recent study in major depression patients in a task which incorporates both predictable and unpredictable aversive stimuli (Grillon et al., 2013). In this task, MDD patients exhibited higher baseline startle reactivity as well as greater potentiation during the cue that was predictive (100% contingency) of an aversive event. The increased startle potentiation was associated with symptom chronicity as well as severity. The different results across this study and the present study are unlikely due to differences in symptom severity (mean BDI 26 vs. 29 for present and previous studies, respectively) or treatment (both studies used unmedicated participants). It is possible that the difference between the Grillon et al. study and the present study are due to differences in the chronicity of symptoms, gender demographics (mixed vs. all male sample respectively) and comorbid anxiety (high vs. relatively low respectively). The lack of significant differences in the present study must also

be interpreted with caution given the relatively small sample size in this group ($N = 12$).

The present results suggest differential performance between PTSD and anxiety symptom groups, with general anxiety symptoms being more associated with exaggerated fear responses and PTSD symptoms being specifically associated with a failure to appropriately inhibit fear responses to safety signals and reduced extinction. This differential pattern of results is suggestive of differences at the neurocircuit level. The higher overall responding in the anxiety symptom group may reflect hyperactivity in emotion-generating limbic circuits, consistent with the neuroimaging evidence for heightened amygdala activation to negative provocation in subjects with generalized anxiety (i.e., Rauch et al., 2003). While PTSD has also been associated with limbic system hyperactivity (Shin et al., 2006), neuroimaging studies have shown more pronounced findings of hypoactivation in structures responsible for inhibition of the limbic system, specifically the medial prefrontal cortex (mPFC) and the rostral and dorsal regions of the anterior cingulate cortex (Etkin and Wager, 2007). Further, Milad et al. (2007, 2008) have demonstrated that individuals with PTSD exhibit reduced ability to recall fear extinction (or fear inhibition) 24 h after initial learning, an ability that is dependent upon mPFC activation. Reduced activity of ventromedial prefrontal cortex is also associated with increased potentiation to CS− and reduced extinction of CS+ (Jovanovic et al., 2013). Thus this pattern of hypoactivation in fear inhibition circuits may be reflected in the current results of relatively normal magnitude of fear responses but poor safety-signal learning and reduced extinction in PTSD symptom groups. The present findings also raise the possibility that this task could identify, via differential patterns of response (exaggerated fear response vs. impaired fear inhibition), those who are neurobiologically at risk for developing a certain class of pathology post-trauma. Previous research has suggested that impaired fear extinction may be a marker for increased risk of developing PTSD following a trauma (Guthrie and Bryant, 2006; Pole et al., 2009; Lommen et al., 2013). Future studies may examine whether these phenotypes predict differential treatment responses to pharmacological or behavioral therapies.

Some limitations of the current study must be acknowledged. First, the paradigm was not effective in producing fear-potentiated startle in ~11% of the study participants tested. While this failure resulted in a reduction of sample size, the excluded participants did not appear to differ systematically from the study volunteers as a whole (supplemental Table 1). Second, the study was conducted on a highly screened cohort of active duty Marines and Navy corpsmen, which limited the number of participants displaying psychiatric symptoms of sufficient intensity for inclusion in the symptom groups. Therefore, the number of participants included in the symptom groups is relatively small, particularly the depression group. It is possible that low power may have contributed to the inability to detect significant differences in between the depression and healthy control group. However it is important to note that the present findings of normal fear inhibition and extinction in the depression symptom group replicate previous studies with greater subject numbers (Jovanovic et al., 2009, 2010). Third, the current study did not explicitly examine the effects of trauma or deployment history on fear conditioning and extinction performance, or on psychiatric outcomes. All symptom groups exhibited significantly higher trauma burden severity (i.e., LEC scores) compared to the healthy group, however no differences were detected between PTSD, anxiety and depression symptom groups, suggesting that trauma burden alone is unlikely to explain differences in task performance across the symptom groups. Future analyses will investigate the role of these variables in influencing task performance, as well as their interaction with psychiatric symptoms. Finally, while the symptom groups had significantly higher scores on their respective assessment measures relative to the other groups (Table 1), all symptom groups also differed from healthy controls across all measures. This elevation across symptom measures speaks to the difficulty of achieving "pure" symptom categories given the large amount of overlap in phenomenology among these conditions. However, the current paradigm was effective in discriminating between symptom classes based on severity, and as whole it appears that the current results have captured differences between groups characterized by predominant symptoms unique to anxiety and PTSD. A final limitation is the use of categorical cutoffs for our symptom groups, which are necessarily arbitrary. However, treating our symptom indicators as quantitative is problematic given our largely healthy sample. Future research in other naturalistic samples may wish to examine quantitative relationships between fear learning indices and symptoms of psychopathology.

In sum, the fear conditioning and extinction paradigm appears to function as anticipated in this active-duty Marine/Navy cohort. Further, the current study represents the first direct comparison of fear conditioning and extinction performance across healthy control, PTSD, anxiety, and depression symptom groups in a fairly homogenous sample. The results point to differential biobehavioral "signatures" associated with distinct symptom groups and may lead toward development of objective markers for classification of psychiatric dysfunction. Future research in this sample will continue to characterize the nature of fear learning abnormalities and examine whether poor learning of safety signals provides a marker of vulnerability to develop PTSD or is specific to symptom state.

Conflict of interest

In the past three years, MAG has received consulting compensation from Abbott, Addex, Cerca, Dart, Lundbeck/Otsuka, Neurocrine, Omeros, Sunovion, Takeda, and Teva, and holds an equity interest in San Diego Instruments. MAG also has research grant support from Intracellular Therapeutics, Johnson & Johnson, NIDA, NIMH, and the U.S. Veteran's Administration VISN 22 Mental Illness Research, Education, and Clinical Center. VBR has received grant funding from Janssen and Omeros. The rest of the authors report no conflicts of interest associated with the current manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.09.030.

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The cumulative effect of different childhood trauma types on self-reported symptoms of adult male depression and PTSD, substance abuse and health-related quality of life in a large active-duty military cohort

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ABSTRACT

History of childhood trauma (CT) is highly prevalent and may lead to long-term consequences on physical and mental health. This study investigated the independent association of CT with symptoms of adult depression and posttraumatic stress disorder (PTSD), mental and physical health-related quality of life (HRQoL), as well as current tobacco consumption and alcohol abuse in a large homogenous cohort of 1254 never-deployed, young male Marines enrolled in the Marine Resiliency Study. Independent effects of CT history, number and type of CT on outcomes were analyzed using hierarchical multivariate logistic regression models. Our results suggested dose-dependent negative effect of an increasing number of trauma types of CT on depression, PTSD and HRQoL. Experience of single CT type demonstrated overall weak effects, while history of multiple CT types distinctively increased the likelihood of adult PTSD symptomology (OR: 3.1, 95% CI: 1.5–6.2), poor mental (OR: 2.3, 95% CI: 1.7–3.1) and physical HRQoL (OR: 1.4, 95% CI: 1.1–1.9). Risk for depression symptoms was similar for both single and multiple CT (OR: 2.2, 95% CI: 1.3–3.8 and OR: 2.1, 95% CI: 1.2–3.5 respectively). CT history had no effects on current tobacco use and alcohol abuse. Our study thus provides evidence for substantial additive effect of different CT types on adult mental and physical health with increasing levels of exposure.

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1. Introduction

Experience of early-life stressors is highly prevalent in the general population and constitutes a major public health problem (Edwards et al., 2003; Gilbert et al., 2009; Green et al., 2010). Persistent functional, and epigenetic changes as a sequelae of early trauma could mediate risk for disease in adulthood, and lead to cumulative disadvantages and increased physical and mental morbidity in later life (Kaufman et al., 2000; Nemeroff, 2004; Gilbert et al., 2009; Shonkoff et al., 2009). Especially a higher risk for psychiatric disorders (e.g., depression, posttraumatic stress disorder and other anxiety disorders) and their unfavorable outcomes has been repeatedly associated with a history of childhood trauma (CT) in several retrospective (Heim and Nemeroff, 2001; Maercker et al., 2004; Pirkola et al., 2005; Scott et al., 2011; Nanni et al., 2012) but also prospective studies (Koenen et al., 2007; Wang et al., 2010; Berntsen et al., 2012; Hovens et al., 2012).

Nevertheless, the chronic physical health consequences of childhood adversities may be as substantial as mental health consequences (Goodwin and Stein, 2004; Scott et al., 2011). Prior research suggests an association of CT with cardiovascular, pulmonary and metabolic diseases, chronic inflammatory and pain syndromes,

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frequency of medical consultations and number of medical diagnoses (Dong et al., 2004; Springer et al., 2007; Korkeila et al., 2010; Stein et al., 2010; Tamayo et al., 2010; Scott et al., 2011). In addition, risk behavior patterns such as substance use and especially tobacco and alcohol consumption are considered significantly increased in individuals with experience of CT (Spratt et al., 2009; Khoury et al., 2010; Wu et al., 2010; Strine et al., 2012; Fenton et al., 2013; Fuller-Thomson et al., 2013). Consequently, many studies have reported a negative impact of CT on adult general mental and physical health-related quality of life (HRQoL) (Walker et al., 1999; Spertus et al., 2003; Elstad, 2005; Draper et al., 2008; Dube et al., 2010).

However, prior studies have generally focused on smaller, clinical and mostly female samples reporting specific trauma types, namely sexual or physical abuse or larger but heterogeneous community samples. Additionally, previous research has largely failed to control for other variables that have an independent association with physical and mental CT sequelae, such as frequency and type of CT, ethnicity, age, current psychopathology, employment and socioeconomic status (Edwards et al., 2003; Thompson et al., 2004; Pirkola et al., 2005; Finkelhor et al., 2007; Suliman et al., 2009; Mock and Arai, 2010). However, more recently, studies that show an association between CT and adult mental and physical health have tended to appropriately include possible confounds in their analyses enabling better interpretability and generalizability of findings (Chu et al., 2013).

The purpose of this study is to extend outcomes of prior research by examining the independent impact of CT history, type and number of traumas on adult symptoms of depression and PTSD, substance abuse and mental and physical HRQoL in a large, homogenous group of young male Marines, accounting for possible confounds. We hypothesized that CT history would be a robust predictor of all outcome variables and exert a cumulative effect with increasing level of reported traumatization.

2. Methods

Data were collected as part of the "Marine Resiliency Study" (MRS), a large, prospective investigation of active-duty male Marines recruited from four infantry battalions of the 1st Marine Division, CA (VA R&D and UCSD IRB approval #070533) (Baker et al., 2012).

2.1. Subjects

A total of 2585 subjects were recruited and assessed on base prior to scheduled deployment. After complete description of the study, written informed consent was obtained. To increase the homogeneity of our cohort and to reduce possible confounds due to prior deployment, only Marines without a prior deployment history were included in the current analyses ($n = 1254$; mean age 21.5 ± 2.4 years; range: 18–43). There were no other exclusion criteria. The demographic and psychometric characteristics of the study sample are presented in Table 1.

2.2. Measures

History of childhood maltreatment was assessed with the Childhood Trauma Questionnaire (CTQ) (Bernstein and Fink, 1998). The five CTQ subscales (emotional abuse; physical abuse; sexual abuse; emotional neglect; physical neglect) sum to a total CTQ score (range: 25–125). Presence of specific trauma types (yes/no) was determined by meeting a threshold of moderate maltreatment severity as indicated by existing guidelines and prior research (cut-off scores: emotional abuse: ≥13; physical abuse: ≥10; sexual abuse: ≥8; emotional neglect: ≥15; physical neglect: ≥10) (Scher et al., 2001). We categorized our population according to the presence of CT (no CT: none of the CT subcategories experienced; one CT: one CT subcategory experienced; multiple CT: ≥2 CT subcategories experienced).

Depression symptoms was assessed using the Beck Depression Inventory-II (BDI) (Beck et al., 1961). The suggested BDI score of ≥20 was used as a cut-off score indicating at least moderate depression (Beck et al., 1996). Posttraumatic stress symptoms were assessed with the Clinician Administered PTSD Scale (CAPS) (Blake et al., 1995). A DSM-IV diagnosis of PTSD was made using the well-established F1/2 scoring rule (Weathers et al., 1999).

Table 1
Sociodemographic and psychometric data of the total study population ($n = 1254$).

Demographic information		Childhood trauma prevalence	
Age (yrs)	21.5 ± 2.4 (18–43)	Childhood trauma	
Education		No CT	652 (53.7%)
Some High School or GED	59 (4.7%)	One CT	261 (21.5%)
High School Diploma	795 (63.9%)	Multiple CT	302 (24.9%)
Some College	328 (26.4%)	2 CT	138 (11.4%)
College/Masters Degree	62 (5.0%)	3 CT	78 (6.2%)
Marital status		4 CT	73 (5.8%)
Never married	943 (75.5%)	5 CT	13 (1.0%)
Married	298 (23.8%)	Emotional abuse	147 (12.0%)
Divorced/separated/widowed	9 (0.7%)	Physical abuse	392 (32.1%)
Race		Sexual abuse	76 (6.2%)
Black/African American	67 (5.5%)	Emotional neglect	240 (19.7%)
Caucasian	1030 (83.8%)	Physical neglect	282 (23.0%)
Other	131 (10.7%)		
Tobacco use	708 (56.8%)		
Psychometric data			
BDI total score	4 (0–51)	SF-12 MCS	50.5 (8.8–69.7)
Depression	100 (8.0%)	Poor mental health	612 (49.5%)
CAPS total score	10 (0–101)	SF-12 PCS	55.9 (21.6–72.0)
PTSD	55 (4.4%)	Poor physical health	618 (50.0%)
AUDIT total score	9 (0–33)	CTQ total score	36 (25–102)
Alcohol abuse (AUDIT level ≥ 2)	534 (42.9%)		

Descriptive statistics are given as mean ± SD for normally distributed continuous variables, median values (min–max) for skewed continuous variables and total numbers and proportions (%) of valid answers (excluding N/A) for categorical variables, as required. Values in categorical variables are reported for presence of characteristic. AUDIT: Alcohol Use Disorders Identification Test; CT: Childhood trauma; CTQ: Childhood Trauma Questionnaire; multiple CT: ≥2 CT subcategories experienced; BDI: Beck Depression Inventory-II; CAPS: Clinician Administered PTSD Scale; PTSD: Posttraumatic Stress Disorder; SF-12: 12-Item Short Form Health Survey; PCS: Physical Component Score: MCS: Mental Component Score.

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Table 2
Sample characteristics according to self-reported childhood trauma history.

Variables	No CT ($n = 652$)	One CT ($n = 261$)	Multiple CT ($n = 302$)	No vs. One CT	No vs. Multiple CT
				Sig.	
Age (yrs)	21.5 ± 2.5	21.4 ± 2.1	21.4 ± 2.2	ns	ns
CTQ total	31 (25–46)	40 (30–55)	56 (35–102)	**<.001**	**<.001**
BDI total	3 (0–40)	5 (0–44)	7 (0–51)	**<.001**	**<.001**
CAPS total	7 (0–74)	13 (0–80)	17 (0–101)	**<.001**	**<.001**
SF-12 MCS	52.0 (15.5–66.5)	49.9 (8.8–66.4)	47.4 (15.2–62.0)	**<.01**	**<.001**
SF-12 PCS	56.1 (32.5–69.3)	56.1 (40.0–69.4)	55.6 (34.9–72.0)	ns	**<.05**
AUDIT total	8 (0–29)	9 (0–33)	10 (0–28)	ns	**<.05**
Depression	31 (5.0%)	28 (12.0%)	35 (13.1%)	**<.01**	**<.001**
PTSD	14 (2.2%)	12 (4.6%)	25 (8.3%)	**<.05**	**<.001**
Tobacco use	383 (58.8%)	145 (55.6%)	165 (54.8%)	ns	ns
Alcohol abuse	272 (41.8%)	116 (44.4%)	136 (45.2%)	ns	ns
Poor mental HRQoL	264 (40.7%)	137 (53.3%)	191 (63.9%)	**<.01**	**<.001**
Poor physical HRQoL	300 (46.3%)	128 (49.8%)	168 (56.2%)	ns	**<.01**

Descriptive statistics are given as mean ± SD for normally distributed continuous variables, median values (min–max) for skewed continuous variables and total numbers and proportions of valid samples (%) for categorical variables, as required. Values in categorical variables are reported for presence of characteristic (tobacco use, alcohol abuse, depression, PTSD, poor mental and physical health). Statistically significant *p*-values are bolded.
CT: Childhood trauma; CTQ: Childhood Trauma Questionnaire; multiple CT: ≥2 CT subcategories experienced; BDI: Beck Depression Inventory-II; CAPS: Clinician Administered PTSD Scale; SF-12: 12-Item Short Form Health Survey; MCS: Mental Component Score; PCS: Physical Component Score; PTSD: Posttraumatic stress disorder; ns: non-significant.

Alcohol consumption was self-reported on the Alcohol Use Disorders Identification Test (AUDIT) (Saunders et al., 1993). We categorized participants into four levels of hazardous drinking severity, consistent with prior literature (0–7: level 1, 8–15: level 2, 16–19: level 3, 20–40: level 4). An AUDIT level ≥2 indicates hazardous drinking and has been used as a cut-off to define alcohol abuse (Babor et al., 2001). Tobacco use status (smoking 1 cigarette or chewing tobacco 1 ×/day or more) has been assessed as a dichotomous variable (yes/no) as in prior studies (Covey and Tam, 1990). Demographic information has been assessed by MRS study questionnaires.

Physical and mental HRQoL were assessed with the Medical Outcome Study 12-Item Short Form Health Survey (SF-12) (Ware et al., 1996). The SF-12 uses US General population means and standard deviations to provide standardized, empirically derived physical (PCS) and mental health (MCS) composite summary scores ranging from 0 to 100 with lower scores representing poorer health functioning. The PCS assesses physical limitations, difficulties in self-care, role performance, physical and social activities, etc., while the MCS assesses psychological distress and social and role limitations due to emotional problems. MCS and PCS median values were used to categorize individuals with an MCS and PCS total score below (poor HRQoL) or above median score of the assessed population, similar to prior studies (Weber et al., 2005; Draper et al., 2008).

2.3. Statistical analysis

We compared variable differences using chi-square tests, independent-sample *t*-tests and Mann–Whitney *U* tests as appropriate. In order to investigate correlations between number of CT experienced and AUDIT, CAPS, BDI, SF-12 PCS and SF-12 MCS total scores, psychometric scores were log transformed for parametric analysis. Relationships were investigated using the Pearson product–moment correlation coefficient *r*. Separate univariate logistic regressions were performed to assess the impact of various factors on the likelihood of depression and PTSD diagnosis, poor mental and physical health, as well as tobacco consumption and alcohol abuse (*cf.* Table 3). Multivariate logistic regression was performed to determine the independent association of CT level (single or multiple), number of CT types reported and CT type on outcomes respectively, after controlling for significant confounders (*cf.* Table 4, Legend) as shown in the prior univariate analyses. Finally, we verified that all our statistically significant results from the multivariate logistic regressions were in fact significant by

Table 3
Univariate associations between various predictive factors and adult health outcomes in the total sample ($n = 1254$).

Predictors	OR (95% CI)					
	Depression	PTSD	Poor mental health	Poor physical health	Tobacco use	Alcohol abuse
Age	.94 (.86–1.04)	.97 (.87–1.10)	**.95** (.90–.99)*	1.00 (.95–1.05)	1.02 (.97–1.07)	**1.05** (1.00–1.10)*
Tobacco use	1.15 (.76–1.75)	1.46 (.83–2.57)	.96 (.77–1.21)	1.06 (.85–1.33)	–	**4.17** (3.26–5.34)***
Alcohol abuse	**1.70** (1.13–2.57)*	1.30 (.76–2.24)	**1.34** (1.07–1.68)*	1.04 (.83–1.30)	–	–
Depression	–	**11.32** (6.33–20.24)***	**18.56** (8.06–42.72)***	.94 (.62–1.41)	–	–
PTSD	–	–	**4.86** (2.43–9.74)***	**2.13** (1.20–3.79)*	–	–
Poor physical health	–	–	.80 (.64–1.01)	–	–	–
Number of CTs	**1.34** (1.17–1.54)***	**1.54** (1.29–1.84)***	**1.42** (1.29–1.57)***	**1.14** (1.05–1.25)**	.93 (.85–1.02)	1.03 (.94–1.13)
CT level						
One CT	**2.40** (1.41–4.09)**	**2.20** (1.00–4.83)*	**1.66** (1.24–2.22)**	1.15 (.86–1.54)	.87 (.65–1.17)	1.11 (.83–1.49)
Multiple CT	**2.63** (1.59–4.36)***	**4.14** (2.12–8.08)***	**2.57** (1.94–3.41)***	**1.49** (1.13–1.96)**	.85 (.64–1.12)	1.15 (.87–1.51)

Results of significant univariate logistic regression analyses. Results are reported as odds ratios (OR) with the 95% confidence interval (95% CI). Significant OR are bolded; *$p < .05$, **$p < .01$, ***$p < .001$.
Cohort showed a significant association to alcohol abuse (respectively $p = .001$), while race, education level and marital status did not reach statistical significance in any univariate regression analysis (data not shown). Because of the multi-categorical structure of these variables, they were not included in the table in terms of space management. CT: Childhood Trauma; PTSD: Posttraumatic stress disorder; number of CTs: Number of different CT subcategories experienced; multiple CT: ≥2 CT subcategories experienced.

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Table 4
Independent associations between number of childhood traumas types and health-related quality of life, depression and PTSD in adulthood.

Predictors	Depression[a]	PTSD[b]	Poor mental health[c]	Poor physical health[d]
CT level				
One CT	**2.21** (1.28–3.82)**	1.62 (.71–3.68)	**1.44** (1.06–1.95)*	1.14 (.85–1.52)
Multiple CT	**2.06** (1.21–3.51)**	**3.09** (1.54–6.22)**	**2.29** (1.70–3.07)***	**1.43** (1.08–1.89)*
Number of CTs	**1.28** (1.10–1.48)**	**1.41** (1.16–1.70)***	**1.36** (1.23–1.50)***	**1.13** (1.03–1.24)**

Results of multivariate regression analyses controlling for significant confounders. Results are reported as odds ratios (OR) with the 95% confidence interval (95% CI). Significant OR are bolded; *$p < .05$, **$p < .01$, ***$p < .001$. CT: Childhood Trauma; PTSD: Posttraumatic stress disorder; number of CTs: Number of different CT subcategories experienced; multiple CT: ≥2CT subcategories experienced. Multivariate models were all statistically significant and adjusted as follows:

[a] The full model contained alcohol abuse and PTSD diagnosis as additional predictors (Model significance: $p < .001$ for both regression models).

[b] The full model contained depression diagnosis and poor physical health as additional predictors (Model significance: $p < .001$ for both regression models).

[c] The full model contained age, alcohol abuse, depression and PTSD diagnosis as additional predictors (Model significance: $p < .001$ for both regression models).

[d] The full model contained PTSD diagnosis as additional predictors (Model significance: $p = .042$ and $p = .009$ respectively).

performing false discovery rate (FDR) control with a rate equal to 0.1. Statistical analyses were conducted using the Statistical Package for Social Sciences Version 20 (SPSS Inc., Chicago, IL) and R, version 2.15.3. All tests of significance were 2-tailed, and p values < .05 were considered significant.

3. Results

3.1. Prevalence of CT

The prevalence of any self-reported CT type experienced was 46.3%, while 24.9% of participating Marines reported multiple CT types (*cf.* Table 1). Among participants who reported a history of CT, physical abuse was the most common type, followed by physical and emotional neglect, while emotional and sexual abuse were less prevalent. 8.0% of participants met criteria for depression and 4.4% for PTSD. Different CT types have shown varying association with risk of experiencing multiple CT types, with participants reporting history of emotional abuse showing the highest risk (Sexual Abuse: OR: 28.6, 95% CI: 14.1–58.2; Physical Neglect: OR: 43.9, 95% CI: 30.2–63.8; Emotional Neglect: OR: 32.4, 95% CI: 22.2–47.3; Emotional Abuse: OR: 110.3, 95% CI: 50.7–240.1; Physical Abuse: OR: 23.6, 95% CI: 16.8–33.2).

3.2. Correlations

When investigating psychometric measures with respect to the number of CT types reported, a dose-dependent relation was observed, with participants who reported a greater number of CT types also evidencing higher psychopathology scores (*cf.* Fig. 1). Our results suggest significant correlations between number of CT types experienced and higher (log-transformed) psychopathology scores. Number of CT types was weakly, but significantly correlated with the PCS of SF-12 and AUDIT total score ($r = -.069$, $p = .017$; $r = .069$, $p = .039$, respectively) and highly significant with BDI, CAPS and the MCS of SF-12 ($r = .173$, $p < .001$; $r = .243$, $p < .001$ and $r = -.199$, $p < .001$, respectively).

3.3. Overall group differences

We investigated omnibus group differences with respect to the number of CT types experienced. Statistically significant differences were found with respect to psychometric measures for depression, PTSD and the SF-12 MCS, where individuals with history of any CT reported worse scores (data not shown). These differences were more pronounced when comparing individuals with no CT history and multiple CT type history (*cf.* Table 2). In this comparison, the SF-12 PCS and the AUDIT total score also showed statistically significant differences. With respect to tobacco consumption and alcohol abuse we did not find any statistically significant differences across groups.

3.4. Predictors of depression and PTSD symptoms, substance abuse and HRQoL

We performed separate logistic regressions to investigate univariate associations between several predicting factors and our

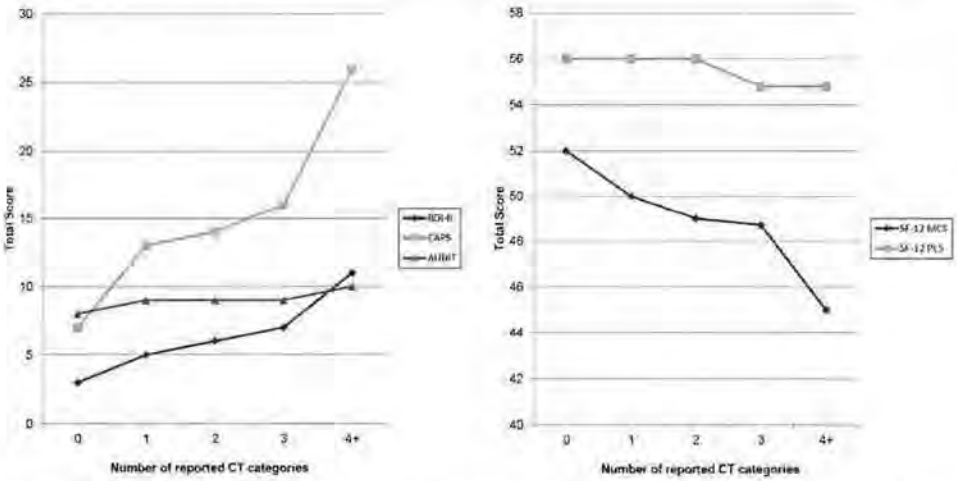

Fig. 1. Median psychometric scores classified by number of reported childhood trauma types. BDI: Beck Depression Inventory-II; CAPS: Clinician Administered PTSD Scale; SF-12: 12-Item Short Form Health Survey. *y*-axis: psychometric scores of the respective questionnaire; *x*-axis: Number of childhood traumas (CT) reported. Participants reporting 4 or 5 CT types have been assessed together as one category (4+), because of their small number.

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main outcomes (PTSD, depression, poor mental and physical HRQoL, tobacco consumption and alcohol abuse). Statistically significant associations are presented in Table 3.

We then performed multivariate logistic regression including all statistically significant factors in order to assess the independent effect of CT level (single CT type, multiple CT types) and number of CT types on the same main outcomes (*cf.* Table 4). Regression models for tobacco consumption and alcohol abuse were not computed because there were no significant associations to CT history in univariate analyses. After controlling for significant confounds, final regression models indicated a dose-dependent relationship between number of CT types experienced and all outcome conditions. History of a single CT type showed a significant independent association only to depression symptoms and poor mental HRQoL, while history of multiple CT types had stronger independent associations to all outcome measures, excluding depression symptoms, which showed similar results to single CT.

Finally, we investigated the independent association of different trauma types on all outcome conditions using the same multivariate models as above to control for confounds. The respective independent associations of emotional, physical and sexual abuse, as well as emotional and physical neglect to all outcome conditions are presented in Table 5.

4. Discussion

This study is to the best of our knowledge the first to investigate the independent association between CT history and self-reported adult depression and PTSD symptoms, substance abuse and mental and physical HRQoL in such a large and homogenous group of young men. In sum, this study provides evidence for a dose-dependent relationship between the number of different CT types experienced and both psychopathology scores and incidence of adult depression and PTSD symptoms, and poor mental and physical HRQoL. There was a significant increase in the independent risk of these four outcomes with increasing number of CT types experienced, with relatively weak independent association of a single CT type, but significant effect of multiple CT types.

The assessed study cohort featured very specific characteristics in terms of sex, age and usual demographic characteristics. In an attempt to control for possible bias, we included only never deployed individuals in our analyses, since prior military deployment has been associated with higher prevalence of psychiatric disorders, substance, alcohol and tobacco consumption, and poorer physical health (Fiedler et al., 2006; Smith et al., 2008; Armed Forces Health Surveillance Center, 2011; Bleier et al., 2011; Thomsen et al., 2011), thus would introduce a confound. The assessed population had higher average rates of pre-deployment PTSD and depression, but similar prevalence of CT history as compared to prior findings in military cohorts (Rosen and Martin, 1996; Wells et al., 2010), although some studies report even higher prevalence for physical and sexual abuse than we observed (Seifert et al., 2011). The prevalence rates of CT and number of CT experienced are comparable to previous reports in large community samples (Edwards et al., 2003; Dube et al., 2010). Our results could therefore possibly represent findings generalizable to the general population of males.

Table 5
Statistically significant independent associations between type of childhood trauma and health-related quality of life, depression and PTSD in Adulthood.

Predictor	Depend. variable	OR (95% CI)
Emotional abuse	Depression	**2.26** (1.31–3.89)**
	Poor mental health	**1.83** (1.25–2.69)**
Physical abuse	PTSD	**1.99** (1.10–3.60)*
	Poor mental health	**1.52** (1.18–1.97)**
Sexual abuse	Depression	**2.21** (1.10–4.46)*
	PTSD	**2.44** (1.07–5.58)*
	Poor mental health	**2.34** (1.37–4.02)**
	Poor physical health	**1.73** (1.07–2.81)*
Emotional neglect	Depression	**1.71** (1.05–2.79)*
	PTSD	**2.58** (1.40–4.76)**
	Poor mental health	**2.65** (1.93–3.65)***
Physical neglect	PTSD	**2.16** (1.18–3.95)*
	Poor mental health	**1.99** (1.49–2.66)***
	Poor physical health	**1.35** (1.03–1.77)*

Results of multivariate regression analyses controlling for significant confounders. Multivariate models were all statistically significant and adjusted as in Table 4. Results are reported as odds ratios (OR) with the 95% confidence interval (95% CI). Significant OR are bolded; *$p < .05$, **$p < .01$, ***$p < .001$.

The major finding of our study is the cumulative adverse effect of different CT types on adult PTSD and depression symptoms, as well as mental and physical HRQoL showing a dose-dependent relationship with increasing number of CT types experienced. While history of a single CT type had at most a weak association with these adult outcomes, the independent association of several experienced CT types showed a definitive effect on adult risk. The severity of health and psychological consequences has often been suggested to be associated with the number of CT types experienced (Walker et al., 1999; Edwards et al., 2003; Huang et al., 2012). Our study is, thus, in accordance to other recent studies providing evidence that an increasing number of different CT types results in higher adult risk for psychiatric symptom complexity and severity, psychiatric comorbidities, poor mental and physical HRQoL, as well as several physical conditions (e.g. heart disease, asthma, diabetes mellitus, arthritis, chronic spinal pain, chronic headache) (Pirkola et al., 2005; Anda et al., 2006; Afifi et al., 2007; Briere et al., 2008; Lang et al., 2008; Suliman et al., 2009, 2010; Scott et al., 2011; Seifert et al., 2011; Sugaya et al., 2012). Here we should note that when investigating history of either one or multiple CT type experiences on depression as a categorical variable we failed to observe a dose-dependent effect between single and multiple CT type on depression symptomology, consistent with some similar published results in depression, and in contrast to most observations in PTSD (Hagenaars et al., 2011; Huang et al., 2012). Interestingly, when we used continuous variables (number of CT types) on depression outcomes or investigated BDI-II total scores, we found a dose dependent depression in relationship with CT. These discrepancies may be an artifact of different statistical approaches and depression cut-offs used in our paper, and across the literature.

The distinct impact of early-life stress may lie on enhanced plasticity mechanisms during this period (Dudley et al., 2011) that lead to persistent functional, and epigenetic alterations and to higher allostatic load over time (Kaufman et al., 2000; Nemeroff, 2004). Experience of CT has been shown to lead to an increased vulnerability to stress, hypothalamic–pituitary–adrenal axis dysregulation, long-lasting alterations in emotional and psychophysiological reactivity, impaired adaptive functioning, malfunction of fear response circuits and distinctive genomic and epigenetic profiles (Heim et al., 2008; Gillespie et al., 2009; Ehlert, 2013; Mehta et al., 2013). Epigenetic modifications mediate the interaction between genetic predisposition and environmental factors and facilitate the response to environmental challenges by regulating functional expression of genes (Jaenisch and Bird, 2003; Bjornsson et al., 2004). They, thus, play a central role in the long-term biological trajectories leading to stress-related disease and may explain inter-individual variation (Yehuda and Bierer, 2009; Klengel et al., 2014). Future studies should utilize larger and collaborative cohorts to implement powerful (epi)genomic approaches towards an identification of early trauma-specific biological pathways (Almli et al., 2014).

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Our study showed no association between CT and alcohol abuse or tobacco consumption, as reported in other studies (Trent et al., 2007; Seifert et al., 2011). This fact might partly rely on the male gender, as well as on group-specific and peer-coherent sample characteristics of our military participants, potentially leading to more intensive consumption patterns than in the general population (Ames et al., 2007; Benjamin et al., 2007; Green et al., 2008; Jones and Fear, 2011). Similarly, neither did demographic characteristics such as age, education, and marital status predict any study outcome. The apparently conflicting findings of significantly higher AUDIT scores in individuals with multiple CT types (*cf.* Table 2) but non-significant impact of CT history on alcohol abuse (*cf.* Table 3) may rely on the used cut-offs for defining alcohol abuse and in the statistically significant, but only weak association of number of CT types and AUDIT score.

Furthermore, our study assessed the association between CT experience and adult physical HRQoL after controlling for mental disorders and mental health status as recommended in prior literature (Springer, 2009; Mock and Arai, 2010). The association between CT and poor physical HRQoL was relatively weak in comparison to our other outcome measures. Taking into account the suggested large gap between early adverse experiences and distinctive biophysiological correlates (Shonkoff et al., 2009), this might in part be explained by the young age and physically active status of our cohort. Overall, military service has been associated with vigorous regular physical exercise, reductions in fat tissue and higher intake of fruits and vegetables than in the general population, while Marines incorporate even higher levels of physical activity in their daily routine than general military population (Warber et al., 1997; Harrison et al., 2000; Headquarters United States Marine Corps, 2002; Mikkola et al., 2009). This, thus, might also explain our observation of the lack of correlation between depression and poor physical HRQoL, in contrast to that described elsewhere (Harder et al., 2011; Aversa et al., 2012).

When investigating the independent association of different CT types on our outcome parameters separately, it is worth mentioning that all five types of CT were significant associated with poor mental HRQoL. That means that independent of CT type, experience of CT led to an increased risk of reporting poor mental HRQoL. On the other hand, our results partly contradict prior studies reporting that mainly non-sexual abuse (e.g. physical and emotional abuse) is related to physical HRQoL (Afifi et al., 2007; Lang et al., 2008; Kelly et al., 2011). Sexual abuse in our study is, actually, the only CT type showing significant independent associations to all outcome parameters, although it did not show stronger association with history of multiple CT types nor was it more prevalent than other CT types. Sexual abuse has been often shown to be more closely associated with a broader range of adult psychiatric symptoms, metabolic risk factors and even epigenetic changes, than other types of CT (Briere and Elliott, 2003; Cougle et al., 2010; Perroud et al., 2011; van Reedt Dortland et al., 2012).

Although our study has major strengths, some limitations merit discussion. Our study did not include information on additional traumatic life events (e.g., loss of parent member, natural disasters, accidental traumas, etc.), parental socioeconomic and educational status or current medical diagnoses. Although we did not assess information on such additional life events in our study, studies that investigated a large number of different childhood stressors (Chu et al., 2013) suggest that interpersonal trauma, as assessed in our study, is of particular importance. Not controlling for the age of traumatization is another important limitation of our study, as there is evidence of its moderating role on adult psychopathology (Maercker et al., 2004; Cutajar et al., 2010; Schoedl et al., 2010). In addition, we cannot discount the fact that responders who reported more severe CT also experienced disproportionately higher cumulative adversity of the same trauma type, as shown in previous studies (Schilling et al., 2008). The retrospective, self-report assessment of CT also limits the ability to infer a causal relationship or the developmental mechanisms between CT exposure and the subsequent onset of adult psychiatric and physical conditions. Retrospective assessment of symptoms may lead to distortion of recollections or bias due to current symptoms (Southwick et al., 1997) and subjective assessment of traumatic experiences could introduce bias and distortions related to cognitive barriers (i.e. fear of stigma, warrior ethos, criticism, etc.) and adaptive denial coping mechanisms (Hoge et al., 2004; Nash, 2007). Furthermore, our study assessed an active duty, mostly non-treatment seeking military cohort, with a relatively small degree of variance in self-reported levels of depressive and PTSD symptoms, introducing an inherent limitation in analysis and interpretation of results. Finally, a confounding factor possibly leading to weaker associations could be the strictly male gender cohort, as many studies have shown stronger CT effects in women (Thompson et al., 2004). This fact, in addition to the overall young age and physically active status of our investigated population, suggests that our results could possibly be yet more robust in an older, mixed-gender participant group of normal physical activity status.

Nevertheless, despite its retrospective nature, our study is consistent with current literature on the role of early stress in diverse chronic mental and physical conditions. Taken together, our results support voluminous prior literature indicating an independent association between childhood trauma and subsequent adult depression, PTSD, as well as poor mental and physical HRQoL (Kessler and Magee, 1993; Young et al., 1997; Walker et al., 1999; Widom, 1999; Heim and Nemeroff, 2001; Spertus et al., 2003; Chapman et al., 2004; Maercker et al., 2004; Nemeroff, 2004; Elstad, 2005; Anda et al., 2006; Koenen et al., 2007; Schilling et al., 2007; Draper et al., 2008; Zlotnick et al., 2008; Scott et al., 2011; Nanni et al., 2012; Chu et al., 2013) and converge with neurobiological evidence of the effect of childhood stress on the body and brain (Glaser, 2000; Nemeroff, 2004).

5. Conclusions

The long-term effects of CT may be conceptualized as a common developmental risk factor triggering a health-related risk cascade with high public health impact. Our results support the theory that CT may increase the risk of mental and physical health status even in early adulthood. Specifically, our study corroborates evidence that CT history is significantly associated with adult PTSD and severe depression symptoms, as well as mental and physical HRQoL in a graded fashion as the number of CT types experienced increases. Recognizing the overlap of different types of childhood adversities is important for understanding its cumulative effect on later-life adjustment (Edwards et al., 2003). Future studies should focus on prospective investigation of potential predictors and mediators, their temporal sequence and combined effects at epidemiological, biological and epigenetic levels, while taking into account the potentially delayed time frame for the expression of their effects. Screening strategies for CT need therefore to be improved. Information about CT history and number of experienced CT would additionally help identify an individual's risk level for disease development and/or help predict response to treatment (Wiersma et al., 2009), as we better understand the relationship between gene and environmental exposures that impacts resilience.

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Author contributions

DGB designed the study. AA, CMN, CJH, SAP, DAB and DGB had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. DGB, JOEP, ACA, CMN and LHA collected the data. AA managed literature searches and wrote the first draft of the paper. All authors revised the draft for important intellectual content. All authors have contributed to, read and approved the final version of the manuscript.

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Conflict of interest disclosure

All authors report no financial relationships with commercial interests.

Disclaimer

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SHORT COMMUNICATION

Characterization of cerebrospinal fluid (CSF) and plasma NPY levels in normal volunteers over a 24-h timeframe

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KEYWORDS
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Blood brain barrier

Summary Neuropeptide Y (NPY) is abundant in mammals, where it contributes to diverse behavioral and physiological functions, centrally and peripherally, but little information is available in regard to NPY cerebrospinal fluid (CSF)/plasma concentration relationships and dynamics. Since plasma NPY levels are commonly used as proxy "biomarkers" for central NPY activity in stress and mental health research in humans this study aims to better characterize the CSF/plasma NPY relationships. Subjects were eleven healthy male volunteers, admitted to the clinical research center for placement of an indwelling CSF catheter, as well as venous catheter, for 24-h collection of CSF NPY (cNPY) and plasma NPY (pNPY) samples. As observed in prior studies, group mean (SE) cNPY concentrations [792.1 (7.80) pg/mL] were higher than pNPY concentrations [220.0 (3.63) pg/mL]. For the eleven normal volunteers who had sufficient common (hourly) pNPY and cNPY data points, analysis of pNPY/cNPY concentration ratios and lagged cross-correlation analysis was completed. Average pNPY/cNPY concentration ratios ranged from .20 to .40 across study subjects, with a mean of .29. pNPY/cNPY cross correlation analyses, computed at varying time lags, were non-significant. An attempt was made to analyze the circadian rhythmicity of NPY secretion, but circadian components were not detectable. Using 24-h data collection, we characterized CSF/plasma NPY relationships, including presentation of evidence of weak CSF and plasma correlations, an important consideration for study design of NPY in stress or mental health.
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1. Introduction

Neuropeptide Y (NPY) acts centrally and peripherally to regulate diverse, endocrine, metabolic, cardiovascular, and immune functions, binding to cloned NPY receptor subtypes (Y1, Y2, Y4, Y5), members of the A1 subgroup of rhodopsin-like G protein-coupled receptors (Hirsch and Zukowska, 2012; Michel et al., 1998). In the central nervous system (CNS), NPY is the most abundant neuropeptide, and is expressed across multiple neuronal systems from the brainstem to the cerebral cortex, including regions that regulate anxiety and fear responses (Michel et al., 1998). Moreover, preclinical pharmacological and transgenic studies have established that NPY promotes anxiolytic behavioral effects, reduces stress responses, fosters recovery from stress, and regulates fear extinction by activating Y1 receptors in amygdala (see Bowers et al., 2012, and Sah and Geracioti, 2012 for review). Human research has provided strong support for the importance of NPY in regulating emotional responses to stress and trauma as well (Bowers et al., 2012). Taken together, these findings indicate that NPY may be a critical regulator of fear and anxiety of relevance to stress associated pathophysiology.

Notwithstanding an interest in NPY as a moderator of stress, and an accumulating literature showing lower concentrations of NPY in plasma or CSF in association with chronic stress, anxiety, and depression, studies of plasma NPY (pNPY) and CSF NPY (cNPY) concentration relationships are few, despite common use of pNPY as a proxy for central NPY activity (see Sah and Geracioti, 2012 for review). Evidence so far indicates that pNPY levels in humans correlate poorly with those in the CNS (Dötsch et al., 1997; Grouzmann et al., 2000; Nam et al., 2001).

Objectives: The objective of this study is to better characterize pNPY/cNPY concentration relationships and biorhythmicity by concurrent measurement of pNPY and cNPY levels in healthy males over a 24-h timeframe.

2. Methods

2.1. Participants

Twelve healthy male civilian study volunteers participated in a serial CSF and plasma sampling study approved by the Institutional Review Board (IRB) of the University of California, San Diego (UCSD) Medical Center and the Research Committee of the San Diego Veterans Affairs Medical Center. One of the volunteers had only a single CSF sample so was excluded from analysis. The 11 remaining participants were mentally healthy, having met study exclusion criteria, which prohibited presence or history of any DSM-IV Axis I mental health disorder or abuse/dependency of alcohol, tobacco or other illicit substances. Likewise, based on a thorough physical examination the volunteers, including blood laboratory tests, chest X-ray and electrocardiogram, all were confirmed to be physically healthy. No subject had weight diverging from the norm ($18 < BMI < 30$), or had a positive urine toxicology screen. None were using any prescribed medications, nor had used either prescribed or over the counter medications for at least five half-lives prior to admission to the clinical research center (CRC). A brief physical examination the afternoon of CRC admission also provided assurance that no subject had acute medical symptoms.

2.2. Procedures

Study volunteers were recruited by verbal, electronic or printed advertisement. Eligible volunteers were invited to the laboratory for an introduction to the study. Following signing of informed consent, the mental and physical health examination procedures described above were administered. Participants who met study criteria were scheduled for admission to the CRC at approximately 5 p.m. the day prior to CSF catheter insertion. From the time of admission until CSF catheter removal, participants remained on a controlled low monoamine diet receiving only standard meals (each 666 calories: 20% protein, 24% fat and 56% carbohydrates) at 7 a.m., 12 p.m. and 6 p.m., in addition to an evening snack of 300 calories at 9 p.m. At 8 p.m. the evening of CRC admission, an indwelling intra-venous (IV) catheter was placed for blood draw. A standard meal was provided, and lights were turned off at 10 p.m. Participants fasted overnight, until approximately 8 a.m., when a 20-gauge catheter was placed in the lumbar subarachnoid space at the L4/5 level, and a second IV line was placed in the antecubital vein of the non-dominant arm for delivery of normal saline solution, infused (100 mL/h) throughout the experiment. At 11:00 a.m., approximately 3 h after CSF catheter placement, the indwelling catheter was attached to an infusion pump for continuous CSF withdrawal at a constant rate of approximately 2 mL/h (48 mL/24 h) into test tubes resting in ice for 24-h. The test tubes were processed each half-hour for storage at −80 °C until immediately before assay. Blood was withdrawn into EDTA-coated tubes at the same intervals and immediately processed in a refrigerated centrifuge prior to storage. Vital signs were monitored hourly during the CSF and plasma sampling, while each subject was awake. Subjects were encouraged to maintain a regular sleep time at around 10 p.m. during each night of study participation, while relative silence (no radio, electronic media, or disturbing conversation) was maintained in the study room.

2.3. Measures

2.3.1. Mental health assessments

Documentation of mental status was completed by a trained clinician supervised by Dr. Baker. Information obtained included basic demographic information, family history psychiatric and physical illness, and via unstructured and structured clinical interviews, history and presence of psychiatric diagnoses or co-morbidities. Structured interviews included the Structured Clinician Interview for the DSM-IV-TR Axis I Disorders (SCID-I) and Hamilton-Depression Scale (HDRS) (Hamilton, 1960).

2.3.2. Neuropeptide Y assays

CSF and plasma were stored at −80 °C until assay of available (hourly) samples. Of the 11 volunteers, 10 had (hourly) samples sufficient for analysis of circadian variation and 8 had a sufficient number of common pNPY and cNPY data points for analysis of pNPY/cNPY correlations. Concentration ratios were calculated from the 7 subjects with at least 12

common data points. A direct radioimmunoassay kit (Euro-Diagnostica-ALPCO Diagnostics, Salem, NH), was used for assay of both cNPY- and pNPY-like immunoreactivity. The highly specific and sensitive antibody has <.1% cross-reactivity with NPY-22-36, peptide YY, pancreatic polypeptide, and other neuropeptides. Assay sensitivity was determined to be ~12.81 pg/mL. The intra-assay coefficient of variation (CV) was 4.7 ± .3% and the inter-assay CV was 8.4 ± .8%.

2.3.3. Statistics

Statistical analyses were conducted using R, version 2.14.2. An error probability of $p \leq .05$ was accepted as statistically significant. Individual NPY time series were displayed in $x-y$ plots and inspected for patterns and outliers. Group means and CSF/plasma ratios were computed. Time ordered relationships between cNPY and pNPY were analyzed for cross-correlation, computed at various time lags covering the 24-h period, by leading or lagging the concentration–time series of cNPY relative to pNPY. A statistically significant cross correlation was declared if a t test was significant at $p \leq .05$. An attempt was made to analyze the circadian rhythmicity of NPY secretion by utilizing a linear mixed-effects model to produce a harmonic analysis (Klemfuss and Clopton, 1993) wherein the joint effects cosine curves representing 24 h and 12 h periods were fit to the NPY data.

3. Results

The mean (SE) age of the eleven healthy volunteers was 30 (1.97) years; age range 21–42 years. Mean body mass index (BMI) was 23.9 (1.60); range 16.5–31.0. Hemodynamic measurements were collected on each subject during waking hours (average 14 observations per subject, range 11–16 observations). Mean (SE) pulse rate was 66.6 (1.10) beats per minute and mean (SE) systolic and diastolic means (SE) were 125.7 (1.56), and 63.9 (1.05), respectively. Mean arterial pressure (MAP) was 84.5 (1.15).

3.1. Mean CSF and plasma NPY concentrations

Group mean (SE) cNPY was 792.1 (7.80) pg/mL. Nine of 11 subjects had sufficient plasma for calculation of group mean (SE) pNPY, which was 220.0 (3.63) pg/mL. There were no significant correlations between either mean cNPY or pNPY

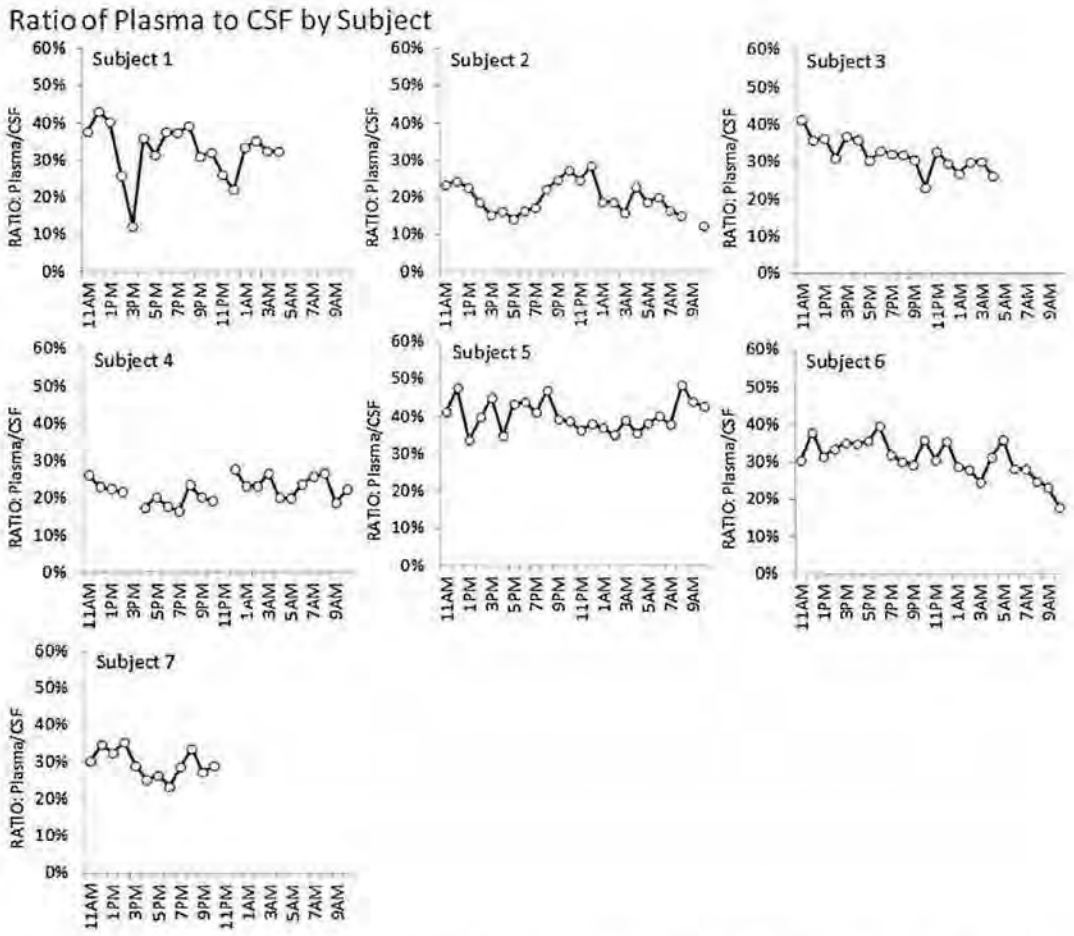

Figure 1 This figure represents pNPY/cNPY concentration ratios for the seven individual study volunteers. The circles represent the pNPY/cNPY ratio at each hour of a 24-h data collection timeframe, where both pNPY and cNPY are available for calculation of pNPY/cNPY ratio.

and any personal or physiological trait, e.g. age, BMI, or any hemodynamic measure.

3.2. Plasma/CSF NPY ratios

Individual average pNPY/cNPY concentration ratios ranged from .20 to .40 across all of the subjects (Fig. 1). The mean (SE) pNPY/cNPY ratio for these subjects was .29 (.007). Ratios were relatively specific for each of the individuals and constant across the 24-h time-frame. No significant correlation between mean pNPY/cNPY concentration ratio and any personal or physiological parameter was observed.

3.3. CSF/Plasma NPY correlations

Lagged cross-correlation analyses showed no statistically significant cross-correlations between cNPY and pNPY (Fig. 2).

3.4. CSF/Plasma NPY variation across the 24-h timeframe

The mean cNPY increase over the 24-h data collection timeframe was 4.60 pg/h. In contrast, no positive or negative linear trend was observed for pNPY. After accounting for the detectable linear trend observable for cNPY, circadian rhythmicity of cNPY and pNPY secretion were analyzed; neither cNPY nor pNPY circadian components were statistically significant, thus we were unable to replicate prior findings suggestive of a pNPY octohoran rhythmicity (Löckinger et al., 2004).

4. Discussion

Based on the individual NPY concentration time-series of our study, group mean CSF NPY values were higher than NPY values observed in plasma, comparable to concentrations previously reported (Dötsch et al., 1997; Grouzmann et al., 2000; Nam et al., 2001). The primary source of plasma NPY is from postganglionic nerve fibers, although NPY can be found in the adrenal medulla and platelets as well (Hirsch and Zukowska, 2012; Takiyyuddin et al., 1994). In the brain, NPY is highly expressed within the hypothalamic nuclei (paraventricular, arcuate), amygdala, hippocampus, septum, and neocortex, but the primary source of CSF NPY has not been clearly delineated (Michel et al., 1998).

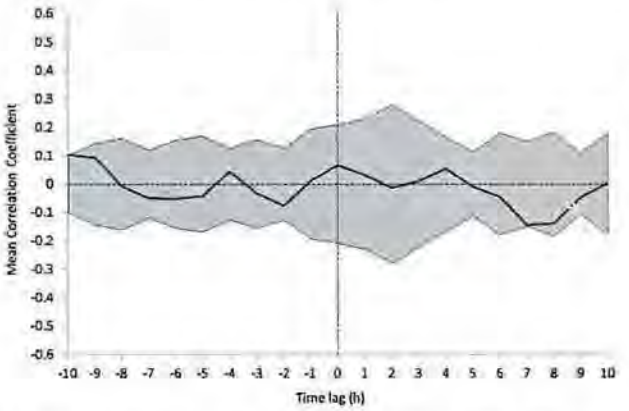

Figure 2 Cross-correlation analysis of cNPY and pNPY concentrations in eight normal male study participants. The *solid line* represents the mean of the individual values of correlation coefficients R_x for each subject at lag time x. The shaded area corresponds to the critical values for statistical significance at the .05 level using a t-test for the cross correlations at each lag. At any lag time, a significant correlation would be represented by falling of the *solid line outside the shaded area*.

Interestingly, the heritability of NPY levels in plasma and CSF is similar, and relatively high. Genetic factors account for about 66% of NPY concentration variance in CSF, in contrast to the much lower heritability of some other CSF peptides, such as corticotropin-releasing hormone (CRH) (Berrettini et al., 1988). Similar genetic heritability is observed for NPY levels in plasma (Zhang et al., 2012). Genetic variation in human NPY haplotypes predicts brain NPY expression, plasma NPY concentrations, and amygdalar activation in response to threat-related facial expressions (Zhou et al., 2008).

Given production of NPY both in the CNS and periphery, pNPY/cNPY concentration ratios are dependent upon a number of factors: rates of production, degradation in both compartments, as well any potential transport across the blood brain barrier (BBB). While a rodent study suggests that NPY may pass from the periphery to the brain via non-saturable transport this situation has not been studied in humans (Kastin and Akerstrom, 1999). Our data could suggest that underlying factors regulating each individual's pNPY/cNPY ratios are relatively constant over a day-long timeframe and that NPY concentration ratios vary over a relatively narrow range (.20 to .40), at least across the individuals tested.

Interestingly, we observed a positive linear trend in NPY concentration from the beginning to the end of the 24-h data collection timeframe. A potential explanation would be progressively enhanced secretion in response to the stress of the procedure. This possibility would be consistent with preclinical research showing that NPY expression in the amgydala increases following stress, and that NPY expression is abnormally low in the amygdala and hippocampus in an animal model of PTSD (Cohen et al., 2012). It has not yet been tested as to whether the positive linear trend in a time-series NPY study co-varies by genotype or by diagnosis.

We detected no statistically significant NPY circadian rhythmicity, either in CSF or plasma, thus were unable to replicate prior findings suggestive of pNPY octohoran rhythmicity (Löckinger et al., 2004). However, this may have been a result of study limitations such as the small number of subjects, or study-related modifications of participant meal and bedtime schedules that interfered with NPY circadian rhythmicity.

Despite relatively constant plasma to CSF NPY ratios within subjects, levels of NPY from CSF and plasma collected concurrently show no cross-correlation across compartments at any time lag. Thus use of NPY assays in studies of humans should be undertaken with caution; plasma NPY levels are unlikely to be accurate proxy markers of CNS NPY activity at any moment in time.

Lastly, given that NPY modulates cardiovascular adaptation to stress with opposite effects in the CNS and periphery, we were interested in NPY concentration relationships and

hemodynamic measures, but observed no significant relationships between any hemodynamic measure and NPY levels or ratios in this small sample set (Hirsch and Zukowska, 2012; Sah and Geracioti, 2012).

In conclusion, there is strong interest in NPY as a moderator of resilience to emotional stress, thus increased research involving measurement of NPY levels in humans with mental disorders. Our 24-h study in normal volunteers indicates the need for caution in using pNPY to predict cNPY or brain NPY concentrations.

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Conflict of interests statement

All authors declare that they have no financial or conflict of interest.

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Diagnostic Utility of the Posttraumatic Stress Disorder (PTSD) Checklist for Identifying Full and Partial PTSD in Active-Duty Military

Benjamin D. Dickstein, Frank W. Weathers, Abigail C. Angkaw, Caroline M. Nievergelt, Kate Yurgil, William P. Nash, Dewleen G. Baker, Brett T. Litz and the Marine Resiliency Study Team

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Diagnostic Utility of the Posttraumatic Stress Disorder (PTSD) Checklist for Identifying Full and Partial PTSD in Active-Duty Military

Benjamin D. Dickstein[1], Frank W. Weathers[2], Abigail C. Angkaw[3,4], Caroline M. Nievergelt[3,4], Kate Yurgil[3], William P. Nash[5], Dewleen G. Baker[3,4], Brett T. Litz[6,7], and the Marine Resiliency Study Team

Abstract

The aim of this study was to determine optimally efficient cutoff scores on the Posttraumatic Stress Disorder Checklist (PCL) for identifying full posttraumatic stress disorder (PTSD) and partial PTSD (P-PTSD) in active-duty Marines and Sailors. Participants were 1,016 Marines and Sailors who were administered the PCL and Clinician-Administered PTSD Scale (CAPS) 3 months after returning from Operations Iraqi and Enduring Freedom. PCL cutoffs were tested against three CAPS-based classifications: full PTSD, stringent P-PTSD, and lenient P-PTSD. A PCL score of 39 was found to be optimally efficient for identifying full PTSD. Scores of 38 and 33 were found to be optimally efficient for identifying stringent and lenient P-PTSD, respectively. Findings suggest that the PCL cutoff that is optimally efficient for detecting PTSD in active-duty Marines and Sailors is substantially lower than the score of 50 commonly used by researchers. In addition, findings provide scores useful for identifying P-PTSD in returning service members.

Keywords

PCL, CAPS, PTSD, military, Marines, Sailors, subthreshold

One of the most commonly used instruments for assessing posttraumatic stress disorder (PTSD) in the military is the PTSD Checklist (PCL; Weathers, Litz, Herman, Huska, & Keane, 1993), a 17-item self-report questionnaire that has been shown to have excellent psychometric properties (see Wilkins, Lang, & Norman, 2011, for a recent review). However, with the exception of one study, the diagnostic utility of the PCL has not been evaluated in an active-duty military context. Consequently, most research investigating the prevalence of PTSD in the military has relied on diagnostic cutoff scores derived from studies of civilians or veterans with chronic PTSD (e.g., Hoge et al., 2004; Kim, Thomas, Wilk, Castro, & Hoge, 2010; Schneiderman, Braver, & Kang, 2008; Thomas et al., 2010). It is unclear whether these cutoffs generalize to active-duty personnel, particularly given the reluctance service members often have about reporting mental health problems (Hoge et al., 2004; Kim et al., 2010).

Recently, a new iteration of the PCL, the PCL-5, was developed to coincide with the publication of the *Diagnostic and Statistical Manual of Mental Disorders, fifth edition* (*DSM-5*; American Psychiatric Association, 2013). Although the revised measure will ultimately replace the PCL for *DSM-IV* (American Psychiatric Association, 1994), research on the original remains important, as these efforts guide retrospective analysis of archival data, including data collected from service members who deployed throughout Operations Iraqi Freedom, Enduring Freedom, and New Dawn (OIF/OEF/OND). Moreover, until further research is conducted on the PCL-5, therapists may find the PCL for *DSM-IV* a more informative clinical instrument.

Bliese et al. (2008) attempted to redress the problem of the military's reliance on diagnostic cutoffs derived from civilian

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and veteran samples. They collected PCL data from active-duty soldiers 3 months after participants returned from serving in OIF and OEF and validated them against the structured clinical PTSD assessment module of the Mini International Neuropsychiatric Interview (MINI; Sheehan et al., 1998). Their diagnostic utility analyses showed that cutoff values between 30 and 34 were the most efficient for detecting MINI-based PTSD cases, an important finding given the substantially higher cutoff of 50 found to be optimally efficient in previous studies with Vietnam War veterans (Forbes, Creamer, & Biddle, 2001; Weathers et al., 1993).

However, two methodological aspects of Bliese et al.'s (2008) study may limit the generalizability of their findings. First, the use of the MINI as the diagnostic criterion is a potential limitation because it is not widely employed as a measure of PTSD and has not been validated against the Clinician-Administered PTSD Scale (CAPS; Blake et al., 1995), which has excellent psychometric properties and is considered the gold standard for PTSD assessment (Keane, Street, & Stafford, 2004; Weathers, Keane, & Davidson, 2001; Weiss, 2004). Second, Bliese et al. validated the PCL in a sample of soldiers who screened positive for possible behavioral health problems during a first-stage assessment. Nearly half (49.5%) of potential participants screened negative and as a result were not administered the MINI or included in utility analyses. Thus, it is unclear whether the cutoff scores identified are useful for identifying cases of PTSD in unrestricted samples of active-duty personnel (e.g., in screening contexts or epidemiological studies).

In this study, we sought to expand on the findings reported by Bliese et al. (2008) and fill gaps in the associated literature. To do so, we evaluated the diagnostic utility of the PCL in a large cohort of active-duty Marines and Sailors who were deployed to OIF/OEF. Using the CAPS for *DSM-IV* as the criterion, our aim was to determine optimally efficient cutoff scores for diagnosing PTSD. We chose to focus primarily on diagnostic efficiency, as it is a measure of test performance that represents a balance between high sensitivity (which minimizes the likelihood of false negatives) and high specificity (which minimizes the likelihood of false positives) and can be interpreted as the extent to which test results are accurate overall. Whereas a highly sensitive test is most appropriate for screening purposes (100% sensitivity ensures that all positive cases are identified), and a highly specific test is most appropriate for diagnostic confirmation (100% specificity ensures that a positive test is never wrong), a highly efficient test maximizes the overall accuracy and is thus optimal for differential diagnosis. Given service members' concerns about reporting mental health problems, as well as the relatively low cutoffs found by Bliese et al. (2008), we hypothesized that the optimally efficient cutoffs identified in our study would fall below 50.

In addition to determining PCL cutoffs for full PTSD, we also were interested in determining cutoffs for partial PTSD (P-PTSD). Also referred to as subthreshold or subsyndromal PTSD (Mylle & Maes, 2004; Zlotnick, Franklin, & Zimmerman, 2002), P-PTSD is associated with increased risk for delayed PTSD and comorbid disorders (Marshall et al., 2001; Pietrzak, Goldstein, Malley, Johnson, & Southwick, 2009), as well as higher levels of functional impairment, including occupational, relationship, and health problems (Breslau, Lucia, & Davis, 2004; Mylle & Maes, 2004; Pietrzak et al., 2009; Zlotnick et al., 2002). Furthermore, returning veterans with P-PTSD report similar rates of suicidal ideation, hopelessness, and aggressive acts as those with full PTSD (Jakupcak et al., 2007). Given the functional impairments associated with P-PTSD, the military considers it a stress injury, a psychological state falling between normal levels of stress reactions and stress-related illnesses such as PTSD (e.g., the Navy-Marine Corps Stress Continuum Model; Nash, 2011). It is assumed that if stress injuries are not adequately addressed, performance and mission-readiness are compromised. Because satisfactory recovery requires a combination of institutional support, social support, and formal intervention, it is critical that P-PTSD be accurately identified among service members (Litz, Steenkamp, & Nash, in press). To date, however, there is no consensus definition for P-PTSD. Thus, to examine the impact of adopting different definitions of P-PTSD, we employed lenient and stringent definitions and conducted separate diagnostic utility analyses for each.

Method

Procedure

Data were collected as part of the Marine Resiliency Study (MRS), a longitudinal project examining risk and resiliency factors among active-duty U.S. Marines and Sailors deploying to OIF/OEF. Assessments were conducted prospectively at one of two Marine bases located in southern California. Participation entailed completing a comprehensive battery of biopsychosocial measures, including self-report forms and structured diagnostic interviews (see Baker et al., 2012, for an overview of study procedures). Interviews were administered by master's and doctoral level clinicians with extensive psychological assessment training, and a subset of interviews were independently rated by a second study clinician to evaluate interrater reliability. The data analyzed in the present study were collected from four separate cohorts of Marines and Sailors who completed assessments at approximately 3 months postdeployment (i.e., 3 months after participants returned to the United States). Data collection took place between June, 2009 and September, 2011. Written informed consent was obtained from all study participants, and the Institutional Review Boards at the University of California, San Diego, the San Diego and Boston VA Healthcare

Table 1. Characteristics of the Study Sample.

Measure	*n*	%	*M*	*SD*
Age (years)			23.36	3.40
Race/ethnicity				
American Indian/ Alaskan	15	1.5		
Asian American	26	2.6		
Black/African American	37	3.7		
Hawaiian/Pacific Islander	13	1.3		
Hispanic/Latino	233	23.1		
White	653	64.8		
Multiracial/other	31	3.1		
Military rank				
E1-E3	664	65.5		
E4-E5	283	27.9		
E6-E9	42	4.1		
O1-O3	20	2.0		
Warrant or field officer	4	0.4		
Education				
Some high school	26	2.6		
GED	20	2.0		
High school diploma	621	61.5		
Some college	284	28.1		
Associates degree	20	2.0		
4-year college degree	33	3.3		
Master's degree	5	0.5		
Marital status				
Never married	588	58.0		
Married	389	38.4		
Divorced/separated	36	3.6		
Time in military (years)			3.14	2.75
Previously deployed	440	43.5		
Number of deployments			1.46	0.96

Note. $N = 1,016$. Valid percentages reported.

Systems, and the Naval Health Research Center, San Diego approved all study procedures and materials.

Participants

Participants were 1,016 male, active-duty U.S. Marines and Sailors who had recently returned from serving in OIF/OEF (women were not included in our sample as all participants were members of infantry battalions). Assessments were conducted at approximately 3 months postdeployment; on average, participants had been back in the United States 98.58 days ($SD = 14.50$). Participant demographics are presented in Table 1.

Measures

Self-reported PTSD symptoms were assessed immediately following completion of the CAPS interview using the PTSD Checklist–Specific Version (PCL-S; Weathers, Litz, Herman, Huska, & Keane, 1993), a 17-item measure assessing each symptom of PTSD contained in the *DSM-IV*. The PCL-S is one of three versions of the PCL, which differ only in terms of the index event to which symptoms are linked. Unlike the PCL civilian and military versions (PCL-C and PCL-M), which instruct individuals to link symptoms to "stressful experiences" and "stressful military experiences" respectively, the PCL-S is linked to a specific index event (in this case, the same index event that was used during participants' CAPS interviews). Consequently, the PCL-S may be more likely to capture PTSD and discriminate it from other forms of psychopathology than the PCL-C and PCL-M (Wilkins et al., 2011). The PCL-S has strong psychometric properties and is widely used by trauma researchers and clinicians (e.g., Wilkins et al., 2011). Internal consistency was high in the current sample, with a Cronbach's alpha of .90.

In addition, anxiety and depressive symptoms were assessed using the Beck Anxiety Inventory (BAI; Beck, Epstein, Brown, & Steer, 1988) and Beck Depression Inventory–II (BDI-II; Beck, Steer, & Brown, 1996). These measures were included in the diagnostic utility analyses to provide a comparison for the performance of the PCL. The BAI and BDI-II are widely used and have been extensively tested and validated (e.g., Beck & Steer, 1991; Beck & Steer, 1993; Dozois, Dobson, & Ahnberg, 1998; Hewitt & Norton, 1993; Osman, Kopper, Barrios, Osman, & Wade, 1997). Both the BAI and BDI-II demonstrated high internal consistency in the current sample, each with an alpha of .92. To examine levels of functioning across diagnostic groups (i.e., full PTSD and lenient and stringent P-PTSD), we administered the World Health Organization–Disability Assessment Schedule II–Short Version (WHODAS-II Short Version; Smith & Epping-Jordan, 2000), a 12-item measure assessing a wide range of functional domains, including social and occupational functioning. Internal consistency was high, with an alpha of .91.

PTSD symptoms were also measured using the CAPS (Blake et al., 1995), a structured diagnostic interview assessing all *DSM-IV* criteria for PTSD. The CAPS assesses the frequency and intensity of PTSD symptoms on separate 5-point (0-4) rating scales. Consistent with previous recommendations (e.g., Weathers, Ruscio, & Keane, 1999), symptoms were considered present if they had occurred at least once within the past month and with at least moderate intensity (i.e., the "Frequency 1/Intensity 2" rule). Internal consistency, based on item severity scores (frequency plus intensity), was high, with an alpha of .81. Interrater reliability was previously evaluated in another MRS study using intraclass correlation coefficients and found to be high (see Yurgil et al., 2014).

Three criterion variables were computed for the purposes of this study, full PTSD and stringent and lenient

P-PTSD. For full PTSD, participants needed to endorse a sufficient number of symptoms to satisfy all *DSM-IV* criteria, that is, one criterion B symptom (re-experiencing), three criterion C symptoms (avoidance and numbing), and two criterion D symptoms (hyperarousal). For stringent P-PTSD, participants needed to endorse a minimum of one criterion B, two criterion C, and two criterion D symptoms (i.e., the same criteria as full PTSD save one criterion C symptom). For lenient P-PTSD, participants needed to endorse a minimum of one criterion B symptom plus three criterion C or two criterion D symptoms (i.e., participants did not need to endorse symptoms in all three clusters). Although numerous scoring rules have previously been used to operationalize P-PTSD, the current rules were selected based on their past use in the research literature, as well as their documented association with functional impairment (e.g., Adams, Boscarino, & Galea, 2006; Mylle & Maes, 2004; Pietrzak et al., 2009; Schnyder, Moergeli, Klaghofer, & Buddeberg, 2001). Given evidence that fear, helplessness, or horror are variably reported, and are less commonly endorsed by males in response to traumatic events (Creamer, McFarlane, & Burgess, 2005; O'Donnell, Creamer, McFarlane, Silove, & Bryant, 2010; Karam et al., 2010; Pereda & Forero, 2012), criterion A2 was not factored into the computation of our PTSD variables (criteria A1, E, and F were met by all participants assigned a diagnosis).

Data Analysis

A series of preliminary analyses were conducted to (a) determine the prevalence associated with each diagnostic scoring rule (full PTSD, lenient P-PTSD, stringent P-PTSD) and examine differences in functional impairment across groups; (b) obtain descriptive information for self-report measures (i.e., the PCL-S, BDI-II, and BAI) for purposes of comparison with other populations (i.e., to determine if symptom underreporting may be an issue in the current sample); and (c) obtain a nonparametric smoothing regression curve (i.e., a loess curve; Jacoby, 2000) to examine the relationship between participants' PCL-S scores and their total number of CAPS symptoms met (i.e., determine the extent to which this relationship is continuous). Loess (locally weighted scatterplot smoothing) can be used to fit a regression curve to scatterplot data without a priori specification of shape. SPSS defaults were used for smoothing parameters (an Epanechnikov kernel, 50% of data points incorporated). SPSS version 21.0 was used for all preliminary analyses. No participants were missing data on the PCL, BDI-II, BAI, or CAPS. Kraemer's signal detection methodology (Kraemer, 1987, 1992) was then used to evaluate the utility of the PCL in predicting full and partial PTSD.

Kraemer's approach involves calculation of measures of test performance, including sensitivity, specificity, efficiency, and positive and negative predictive values, as well as corresponding measures of test quality, which are weighted kappa coefficients that adjust for chance agreement between the test and criterion. Measures of test quality are unambiguous, calibrated indicators with endpoints ranging from .00, reflecting chance agreement between test and criterion, to 1.00, indicating perfect agreement. They allow identification of optimally sensitive cutoff scores, which minimize false negatives and thus are ideal for screening; optimally specific cutoffs, which minimize false positive and thus are ideal for confirmatory tests; and optimally efficient cutoff scores, which maximize agreement between test and criterion and thus are ideal for differential diagnosis. In the present study the focus was on optimally efficient tests. Because there are no absolute standards regarding acceptable test efficiency, the BDI-II and BAI were included to provide a basis of comparison. Following Kraemer's (1987) recommend-dation, all cutoffs examined were observed in at least 10 participants.

Results

Prevalence of full PTSD was 4.1% ($n = 42$). As expected, prevalence was higher for stringent P-PTSD (6.2%; $n = 63$) and highest for lenient P-PTSD (11.1%; $n = 113$). To examine differences in functioning across diagnostic groups, we conducted a one-way analysis of variance with Tukey post hoc comparisons. Post hoc comparisons revealed that, relative to participants with no diagnosis, Marines and Sailors in the lenient P-PTSD, stringent P-PTSD, and full PTSD groups scored significantly higher on the WHODAS-II (according to both ANOVA and Kruskal-Wallis tests), suggesting that the diagnostic scoring rules used in the current study are associated with significant functional impairment (see Table 2).

Descriptive information for the PCL-S, BDI-II, and BAI was as follows: participants' mean score on these measures was 22.37 ($SD = 8.00$; range = 17-67), 4.72 ($SD = 6.73$; range = 0-50), and 4.51 ($SD = 7.04$; range = 0-57), respectively. With regards to the frequency of minimum scores, 365 (35.9 %), 366 (36.0%), and 429 (42.2 %) participants reported the lowest possible score on each measure, respectively. Results from a nonparametric smoothing regression curve (Figure 1) demonstrate a continuous, positive relationship between participants' PCL-S scores and the number of PTSD symptoms met on the CAPS.

Signal detection results are presented in Table 3. Across all three diagnostic criteria the PCL demonstrated substantially higher quality of efficiency than did the BDI-II and BAI. With regards to cutoffs, PCL scores of 39, 38, and 33 were found to be optimally efficient for detecting full PTSD, stringent P-PTSD, and lenient P-PTSD, respectively.

Table 2. Means and Standard Deviations of Functioning According to Diagnostic Scoring Rule.

	No PTSD/P-PTSD, *M* (*SD*)	Lenient P-PTSD, *M* (*SD*)	Stringent P-PTSD, *M* (*SD*)	Full PTSD, *M* (*SD*)	*F* (*df*)
n (%)	903 (88.9)	50 (4.9)	21 (2.1)	42 (4.1)	
WHODAS	$13.76\ (4.04)_a$	$15.93\ (5.56)_b$	$18.43\ (8.85)_b$	$17.67\ (6.75)_b$	20.58 (3, 1012)***

Note. PTSD = posttraumatic stress disorder; P-PTSD = partial posttraumatic stress disorder; WHODAS = World Health Organization Disability Assessment Schedule–II Short Version. Means with different subscripts differ significantly at $p < .01$. Valid percentages reported.
*** $p < .001$.

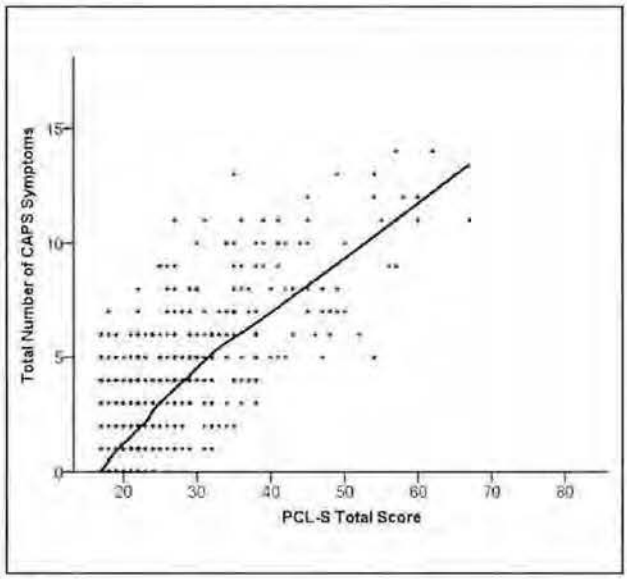

Figure 1. A nonparametric loess smoothing curve depicting the relationship between participants' scores on the PTSD Checklist–Specific Version (PCL-S) and their total number of PTSD symptoms, as determined using the Clinician Administered PTSD Scale (CAPS). Symptoms were considered present on the CAPS if they had occurred at least once within the past month and with at least moderate intensity.

Measures of test performance and quality for PCL cutoffs ranging from 30 to 50 for predicting full PTSD are presented in Table 4.

Discussion

Signal detection analysis was used to examine the diagnostic utility of the PCL among 1,016 MRS participants assessed at 3 months following return from deployment to OIF/OEF. To our knowledge, only one previous study (Bliese et al., 2008) has tested the diagnostic utility of the PCL in an active-duty population. Analyses revealed that a PCL cutoff score of 39 was optimally efficient for identifying full PTSD. Although this score is somewhat higher than the optimally efficient cutoffs (30-34) identified by Bliese et al., it is substantially lower than the cutoff of 50 found in previous studies conducted with Vietnam War veterans (Forbes et al., 2001; Weathers et al., 1993) and reaffirms that different cutoff values are indicated for identifying PTSD among active-duty versus veteran populations.

The lower diagnostic cutoff found in this study may reflect an unwillingness on the part of active-duty service members to report mental health problems due to concerns such as being stigmatized or being denied opportunities for advancement (Gorman, Blow, Ames, & Reed, 2011; Hoge et al., 2004; Kim et al., 2010). Such a response bias could affect scores on both the PCL and CAPS, lowering test scores and prevalence and thereby lowering the optimally efficient cutoff. Consistent with this hypothesis, self-report scores were highly positively skewed, and there was a preponderance of minimum values. Whereas the mean PCL-S score in our sample was 22.37, higher PCL means have been found in other published unrestricted samples, including in Persian Gulf War veterans ($M = 34.77$; Weathers et al., 1993).

Regardless of the reasons for the lower test scores in this sample, it appears that a PCL cutoff of 50 is too high for identifying PTSD among active-duty service members returning from combat. We found a cutoff of 50 to have markedly lower quality of sensitivity relative to other cutoffs (Table 4), indicating that its use would result in a high rate of false negatives (i.e., a large number of unidentified cases). Based on these findings and the findings of Bliese et al. (2008), it appears that a self-reported PCL score in the mid- to upper-thirties is more appropriate for identifying PTSD in this population. It is noteworthy, however, that the level of sensitivity associated with a cutoff score of 39 (Sens = .60) was still relatively low, indicating that its use could result in a considerable number of false negatives. Thus, for screening purposes, a lower cutoff appears indicated.

In addition to identifying optimally efficient cutoff scores for detecting full PTSD, we also sought to identify cutoffs indicative of P-PTSD, a condition associated with functional impairment and increased risk for suicidal ideation (e.g., Marshall et al., 2001). To achieve this, we used two P-PTSD classifications, lenient P-PTSD and stringent P-PTSD, which meaningfully differentiated participants' functional impairment. Cutoffs of 33 and 38 were optimally

Table 3. Diagnostic Utility of Optimally Efficient Cutoff Scores on the PTSD Checklist, Beck Depression Inventory, and Beck Anxiety Inventory for Predicting Full PTSD, Stringent Partial PTSD, and Lenient Partial PTSD Diagnostic Status Based on CAPS F1/I2 Scoring Rule (N = 1,016).

Measure (cutoff score)	Level (%)	Sens	Spec	PPV	NPV	Eff	κ(0)	κ(.5)	95% CI	κ(1)
Full PTSD (Base rate = 4.1%)										
PTSD Checklist total (39)	5.0	.60	.97	.49	.98	.96	.47	.52	.39-.64	.57
Beck Depression Inventory (14)	9.7	.62	.93	.26	.98	.91	.23	.33	.23-.43	.58
Beck Anxiety Inventory (18)	5.5	.31	.96	.23	.97	.93	.20	.23	.11-.35	.27
Stringent P-PTSD (Base rate = 6.2%)										
PTSD Checklist total (38)	6.0	.56	.97	.57	.97	.95	.55	.54	.43-.65	.53
Beck Depression Inventory (14)	9.7	.52	.93	.33	.97	.91	.29	.36	.26-.46	.47
Beck Anxiety Inventory (13)	10.4	.43	.92	.25	.96	.89	.21	.26	.17-.36	.36
Lenient P-PTSD (Base rate = 11.1%)										
PTSD Checklist total (33)	9.7	.58	.96	.66	.95	.92	.61	.57	.48-.65	.53
Beck Depression Inventory (11)	14.9	.50	.90	.38	.94	.85	.30	.35	.27-.43	.42
Beck Anxiety Inventory (13)	10.4	.35	.93	.38	.92	.86	.30	.29	.20-.38	.28

Note. Values rounded to decimal places shown. Level = level of test (i.e., percentage of participants meeting cutoff); PTSD = posttraumatic stress disorder; Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value; Eff = efficiency; CI = confidence interval; κ (0) = quality of specificity; κ (.5) = quality of efficiency; κ (1) = quality of sensitivity. Confidence intervals provided for κ (.5). Measures of test quality are adjusted for chance agreement between the test and criterion. These values range from .00 (chance agreement) to 1.00 (perfect agreement).

Table 4. Diagnostic Utility of Alternative Cutoff Scores on the PTSD Checklist for Predicting Full PTSD Diagnostic Status.

Cutoff	Sens	Spec	PPV	NPV	Eff	κ(0)	κ(.5)	κ(1)
30	.81	.89	.24	.99	.88	.20	.32	.78
31	.81	.90	.26	.99	.90	.23	.35	.78
32	.79	.92	.29	.99	.91	.26	.39	.76
33	.79	.93	.33	.99	.93	.30	.44	.76
34	.79	.94	.35	.99	.93	.32	.45	.76
35	.76	.95	.38	.99	.94	.35	.48	.74
36	.71	.96	.42	.99	.95	.39	.50	.69
37	.67	.96	.43	.99	.95	.41	.50	.64
38	.64	.97	.44	.98	.95	.42	.50	.62
39	.60	.97	.49	.98	.96	.47	.52	.57
40	.52	.97	.47	.98	.96	.45	.47	.50
41	.52	.98	.49	.98	.96	.47	.48	.50
42	.48	.98	.50	.98	.96	.48	.47	.45
43	.45	.98	.53	.98	.96	.51	.47	.43
44	.45	.98	.56	.98	.96	.54	.48	.43
45	.43	.98	.55	.98	.96	.53	.46	.41
46	.36	.99	.56	.97	.96	.54	.42	.34
47	.36	.99	.58	.97	.96	.56	.42	.34
48	.31	.99	.59	.97	.96	.57	.39	.29
49	.29	.99	.60	.97	.96	.58	.37	.27
50	.24	.99	.59	.97	.96	.57	.32	.23

Note. Values rounded to decimal places shown. PTSD = posttraumatic stress disorder; Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value; Eff = efficiency; κ(0) = quality of specificity; κ(.5) = quality of efficiency; κ(1) = quality of sensitivity. Measures of test quality are adjusted for chance agreement between the test and criterion. These values range from .00 (chance agreement) to 1.00 (perfect agreement).

efficient for detecting lenient P-PTSD and stringent P-PTSD, respectively. Given that stringent P-PTSD differed from full diagnostic status by only one Criterion C symptom, it is not surprising that the stringent P-PTSD cutoff was very similar to that suggesting full PTSD.

Clinicians and researchers hoping to apply study results should be mindful of the purpose for which cutoffs are being used. In situations where it is preferred that fewer PTSD or P-PTSD cases go undetected, lower identified cutoff values are indicated. Conversely, in situations where it is

preferred that false positives be minimized, higher cutoffs are recommended. Additionally, individuals wishing to use cutoffs for screening or diagnostic confirmation purposes should be mindful that optimally efficient cutoffs may have relatively poor sensitivity or specificity. As no absolute standards exist regarding "acceptable" sensitivity, it is necessary to adjust cutoffs to meet the needs of particular populations in particular contexts.

There are several important limitations to our study. Although our sample is large and likely representative of Marine Corps and Navy personnel, it is not a stratified random subgroup of Marines and Sailors and does not include female service members, which may affect generalizability. Similarly, our results are based on cutoff scores on the PTSD Checklist–Specific Version (PCL-S), which although highly similar to other versions of the PCL, may be better able to discriminate PTSD from other forms of psychopathology due to differences in instruction set (Wilkins et al., 2011). Of note, administration of the CAPS and PCL was not counter-balanced, and administering the PCL immediately following the CAPS may partly explain why it outperformed the BDI-II and BAI across all diagnostic utility analyses. In addition, a relatively low base rate of PTSD was observed in the current sample, which may have affected diagnostic utility results. Finally, it is important to note that, due to time constraints, comprehensive diagnostic interviews could not be administered, precluding assessment of comorbid psychological conditions.

Limitations notwithstanding, our study makes several important contributions. Most notably, this is only the second study to examine the diagnostic utility of the PCL in an active-duty population, and the first to validate the PCL among active-duty Marines and Sailors. In addition, this is the first study to examine the diagnostic utility of the PCL in any active-duty population using the CAPS, widely considered the gold standard for PTSD assessment, as the diagnostic criterion. As hypothesized, active-duty personnel appeared more likely to underreport PTSD symptoms on the PCL, thus making it important to use lower diagnostic cutoff scores. These findings will help guide indicated prevention efforts within the military and assist researchers and epidemiologists more accurately estimate rates of PTSD and P-PTSD, particularly when conducting archival analysis of data collected from active-duty service members prior to *DSM-5*. Study findings also have important implications for the validation and use of the PCL-5, namely that it be evaluated separately in active-duty and Veteran populations and that screening efforts take into account the possibility of underreporting by using cutoffs demonstrating high sensitivity. Last, while beyond the scope of this article, these findings call attention to the impact of active-duty status on the diagnostic utility of self-report assessment more generally and the extent to which self-report screeners can adequately differentiate service members' diagnostic status. Further research is needed to address these concerns and generate recommendations for optimizing the efficiency of early PTSD and P-PTSD detection.

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Original Investigation

Assessment of Plasma C-Reactive Protein as a Biomarker of Posttraumatic Stress Disorder Risk

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IMPORTANCE Posttraumatic stress disorder (PTSD) has been associated in cross-sectional studies with peripheral inflammation. It is not known whether this observed association is the result of PTSD predisposing to inflammation (as sometimes postulated) or to inflammation predisposing to PTSD.

OBJECTIVE To determine whether plasma concentration of the inflammatory marker C-reactive protein (CRP) helps predict PTSD symptoms.

DESIGN, SETTING, AND PARTICIPANTS The Marine Resiliency Study, a prospective study of approximately 2600 war zone–deployed Marines, evaluated PTSD symptoms and various physiological and psychological parameters before deployment and at approximately 3 and 6 months following a 7-month deployment. Participants were recruited from 4 all-male infantry battalions imminently deploying to a war zone. Participation was requested of 2978 individuals; 2610 people (87.6%) consented and 2555 (85.8%) were included in the present analysis. Postdeployment data on combat-related trauma were included for 2208 participants (86.4% of the 2555 included) and on PTSD symptoms at 3 and 6 months after deployment for 1861 (72.8%) and 1617 (63.3%) participants, respectively.

MAIN OUTCOMES AND MEASURES Severity of PTSD symptoms 3 months after deployment assessed by the Clinician-Administered PTSD Scale (CAPS).

RESULTS We determined the effects of baseline plasma CRP concentration on postdeployment CAPS using zero-inflated negative binomial regression (ZINBR), a procedure designed for distributions, such as CAPS in this study, that have an excess of zeroes in addition to being positively skewed. Adjusting for the baseline CAPS score, trauma exposure, and other relevant covariates, we found baseline plasma CRP concentration to be a highly significant overall predictor of postdeployment CAPS scores ($P = .002$): each 10-fold increment in CRP concentration was associated with an odds ratio of nonzero outcome (presence vs absence of any PTSD symptoms) of 1.51 (95% CI, 1.15-1.97; $P = .003$) and a fold increase in outcome with a nonzero value (extent of symptoms when present) of 1.06 (95% CI, 0.99-1.14; $P = .09$).

CONCLUSIONS AND RELEVANCE A marker of peripheral inflammation, plasma CRP may be prospectively associated with PTSD symptom emergence, suggesting that inflammation may predispose to PTSD.

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Observational studies[1] largely support an association of posttraumatic stress disorder (PTSD) with increased peripheral inflammation, as discussed in a recent review of the overall evidence.[2] For instance, one large cross-sectional community-based study[3] found that patients with PTSD had approximately twice the odds of those without this disorder of elevation in the inflammatory marker C-reactive protein (CRP). Similarly, although some case-control studies[4,5] have had negative or equivocal findings, in most such studies[6-11] PTSD cohorts have had significantly greater plasma levels of CRP or interleukin 6, among other inflammatory markers, than did controls. This association is of prognostic significance because low-grade inflammation is likely involved in the pathophysiology of the metabolic syndrome,[12-14] a major cardiovascular risk factor[15,16]; indeed, PTSD has been found to be associated with this syndrome.[17-24]

It is plausible that the observed association between PTSD and inflammation is due to PTSD-related stress hormone dysregulation leading to alterations in immune, and therefore inflammatory, signaling.[7,25-27] However, given the cross-sectional nature of the evidence at hand, it remains possible that rather than PTSD promoting inflammation, inflammation places individuals at heightened risk for developing PTSD in the setting of trauma. In other words, the direction of causality runs from inflammation to PTSD rather than from PTSD to inflammation.

Service members serving in the Iraq and Afghanistan conflicts endure substantial combat stress and consequent PTSD.[28] The Marine Resiliency Study (MRS) is a prospective field study of approximately 2600 Marines and sailors deployed to Iraq or Afghanistan, during which PTSD severity and various physiological and psychological parameters were determined predeployment and postdeployment, affording an outstanding opportunity to investigate the causal relationship between inflammation and PTSD. In the present study, we determined whether baseline peripheral inflammation, assessed by plasma CRP levels in the MRS, contributes to postdeployment PTSD symptoms, assessed by scores on the Clinician-Administered PTSD Scale (CAPS), adjusting for trauma exposure and other relevant covariates.

Methods

Participants

The MRS is a prospective longitudinal study of biological and neuropsychological modulators of combat stress-related PTSD in Marines.[29] Approval was received and has been maintained since August 2007 from the institutional review boards of the University of California, San Diego, Veterans Affairs San Diego Research Service, and Naval Health Research Center. Participants were recruited from 4 all-male infantry battalions that were imminently deploying to a war zone. Participation was requested of 2978 individuals, of whom 2610 (87.6%) provided written informed consent and were enrolled and given financial compensation. Assessment of the participants began on July 14, 2008, and continued through May 24, 2012. Fifty-five of the enrollees were excluded from the present analysis because they did not deploy with their cohort or withdrew before completing the predeployment visit, so that the number of participants included was 2555 (85.8%). The demographics of these individuals are summarized in **Table 1**.

Data were collected approximately 1 month before a 7-month deployment (baseline; visit 0) and at 1 week, 3 months, and 6 months following the deployment (visits 1, 2, and 3, respectively). Among the 2555 included participants, baseline plasma CRP concentrations were included from 2484 participants (97.2%) and baseline CAPS scores from 2533 participants (99.1%). For the other specific baseline variables used in the present statistical analyses (anthropometrics, psychometrics, and demographics; see below), the number of individuals with included data ranged from 2482 to 2548 (97.1%-99.7%). Data on deployment-related trauma were obtained at visit 1 and were included from 2208 participants (86.4%), visit 2 CAPS scores from 1861 participants (72.8%), and visit 3 CAPS scores from 1617 participants (63.3%).

Measures

The CAPS,[30] a criterion standard PTSD symptom scale, was the primary outcome measure for our analyses because, as a 136-point numeric scale, it would be expected to yield greater discriminant power than the binary outcome of PTSD diagnosis. Trauma exposure occurring during combat was assessed with the Deployment Risk and Resilience Inventory Combat Exposure Scale (CES) (http://www.ptsd.va.gov/professional/assessment/te-measures/ces.asp), and exposure occurring in the aftermath of combat with the Deployment Risk and Resilience Inventory Post-battle Experiences (PBE) scale (http://www.ptsd.va.gov/professional/assessment/deployment/exposure-aftermath-battle.asp). Baseline high-sensitive CRP plasma levels were measured using an enzyme-linked immunosorbent assay (ALPCO Diagnostics). Measures for variables not included in the final regression model are described in the Supplement (eMethods).

Statistical Analysis

The association of our predictors of interest with CAPS was determined using zero-inflated negative binomial regression (ZINBR). A description of this method and the rationale for its choice are in the Supplement (eMethods). Potential confounders were selected for inclusion in regression modeling on the basis of their univariate association at a lenient significance threshold ($P < .20$), with both the outcome (postdeployment CAPS) and the predictor of interest (plasma CRP concentration) (determined by analysis of variance, linear regression, or ZINBR as appropriate). The values for plasma CRP concentrations were skewed and were therefore log transformed before analyses. Ordinal and binomial logistic regression were used to determine the effects of the same predictors as in the final ZINBR model (**Table 2**) on the categorical outcomes at visit 2 of full PTSD (as defined in the *DSM-IV-R*),[31] partial PTSD,[32-34] or no PTSD. Statistical analyses were performed with either SPSS, version 20.0 (IBM) or, for ZINBR, the R statistical package (http://cran.r-project.org). All *P* values reported are 2-tailed.

Table 1. Selected Baseline and Postdeployment Characteristics of Participants[a]

Characteristic	No.	Mean (SD) or %[b]	Median (Range)[c]
Demographics			
Age, y	2548	22.78 (3.51)	21.83 (18-48)
Ethnicity	2534		
Non-Hispanic	1944	76.5	...
Hispanic	590	23.2	...
Race	2503		
European American	2113	82.7	...
African American	119	4.7	...
Asian American	69	2.7	...
American Indian	35	1.4	...
Pacific Islander	38	1.5	...
Mixed	129	5.0	...
Highest educational level	2482		
High school	1645	64.3	...
At least some college	825	32.3	...
Postgraduate	12	0.5	...
Marital status	2538		
Never married	1560	61.1	...
Married	889	34.8	...
Divorced or separated	89	3.5	...
Military characteristics			
Service length, mo	2538	36.29 (24.45)	26.00 (0-324)
Previously deployed	2541	51.3	...
Enlisted	2540	97.4	...
CRP, mg/L	2484	1.93 (3.31)	0.79 (0.03-28.53)
Waist circumference, cm	2533	85.39 (7.62)	84.46 (65.41-123.52)
BMI	2533	27.60 (3.24)	27.42 (18.83-41.41)
Mean arterial blood pressure, mm Hg	2527	90.38 (7.98)	90.00 (64.67-148.33)
AUDIT-C score	2527	5.06 (3.61)	5.00 (0-12)
BAI score			
Visit 0	2519	6.79 (7.85)	4.00 (0-53)
Visit 2	1850	4.79 (7.36)	2.00 (0-57)
Visit 3	1609	4.22 (7.26)	1.00 (0-63)
BDI score			
Visit 0	2526	6.59 (7.67)	4.00 (0-51)
Visit 2	1854	5.05 (6.80)	2.00 (0-54)
Visit 3	1612	4.79 (6.82)	2.00 (0-46)
CAPS score			
Visit 0	2533	14.89 (15.37)	10.00 (0-101)
Visit 2	1861	17.40 (18.01)	12.00 (0-120)
Visit 3	1617	15.41 (17.39)	10.00 (0-107)
PTSD[d]			
Visit 0	2533	4.7	...
Visit 2	1861	6.3	...
Visit 3	1617	5.1	...
CES	2189	13.57 (11.39)	9.00 (0-64)
PBE	2204	5.65 (4.79)	4.00 (0-15)

Abbreviations: AUDIT-C, Alcohol Use Disorders Identification Test-consumption; BAI, Beck Anxiety Inventory; BDI, Beck Depression Inventory; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); CAPS, Clinician-Administered Posttraumatic Stress Disorder (PTSD) Scale; CES, Combat Exposure Scale; CRP, baseline plasma C-reactive protein; PBE, Post-battle Experiences; ellipses, not applicable.

[a] See the Methods section for definition of variables and additional details concerning demographics and military characteristics.

[b] A small proportion of participants did not provide data on demographic traits; therefore, the percentages do not total 100%.

[c] Median (range) values were not determined for values reported as percentages.

[d] Visit 0, baseline; visit 2, 3 months postdeployment; visit 3, 6 months postdeployment.

Results

Choice of Outcomes and Model Covariates

Baseline and postdeployment values of participants for the variables included in the statistical models are listed in Table 1 along with selected additional characteristics. Posttraumatic stress disorder symptoms, assessed by CAPS scores (see the Methods section), increased significantly between the baseline and 3-month postdeployment visits used for our analysis (visits 0 and 2), and then trended back toward baseline in line with findings in a recent systematic review.[35] In contrast to their

Table 2. Zero-Inflated Negative Binomial Regression Model of Postdeployment (Visit 2) CAPS Score in 1719 Participants

Variable	Zero Model		Count Model		Overall P Value[e]
	OR (95% CI)[a,b]	P Value	Fold Change (95% CI)[c,d]	P Value	
Intercept[b,d]	1.25 (0.76-2.05)	.37	10.57 (9.29-12.03)	<.001	
Cohort 1	1.92 (1.08-3.43)	.03	1.03 (0.90-1.19)	.68	<.001
Cohort 2	0.57 (0.36-0.90)	.02	0.94 (0.83-1.07)	.35	<.001
Cohort 3	0.63 (0.41-0.97)	.04	0.78 (0.70-0.86)	<.001	<.001
Cohort 4	0[f]				
CAPS0 score	1.10 (1.08-1.12)	<.001	1.02 (1.02-1.02)	<.001	<.001
CES score	1.03 (1.01-1.05)	.02	1.01 (1.00-1.01)	.001	<.001
PBE score	1.08 (1.03-1.13)	.003	1.04 (1.03-1.05)	<.001	<.001
Log CRP	1.51 (1.15-1.97)	.003	1.06 (0.99-1.14)	.09	.002

Abbreviations: CAPS0, Clinician-Administered Posttraumatic Stress Disorder (PTSD) Scale score at visit 0 (baseline); CES, Combat Exposure Scale; CRP, baseline plasma C-reactive protein; OR, odds ratio; PBE, Post-battle Experiences.

[a] Ratio of approximate odds of nonzero outcome (computed by exponentiating the corresponding coefficient in the regression model and adjusted for the variables listed in the table).

[b] Value for the intercept indicates approximate odds of nonzero outcome at baseline (cohort equals 4 and all other variables have zero values).

[c] Approximate fold change in outcome, in the event of a nonzero outcome (computed by exponentiating the corresponding coefficient in the regression model and adjusted for the variables listed in the table).

[d] Value for the intercept indicates approximate outcome in the event of a nonzero outcome at baseline (cohort equals 4 and all other variables have zero values).

[e] By the likelihood ratio test.

[f] This variable was set to zero because it is redundant.

scores on CAPS, participants' scores on the Beck Anxiety Inventory and Beck Depression Inventory (Supplement [eMethods]) dropped markedly after completion of deployment (Table 1), potentially reflecting the relief experienced by service personnel on return from combat. Thus, the observed postdeployment increases in PTSD symptoms were not attributable to broad psychopathology or general psychological distress.

We included baseline CAPS scores (CAPS0) as a covariate in all statistical analyses of the outcome of visit 2 CAPS (CAPS2) so as to adjust for any differences between participants in CAPS2 that were attributable to preexisting differences in CAPS0; this also adjusted for any effects of baseline PTSD symptoms on the subsequent trajectory of the disorder. In addition to CAPS0, CES and PBE scores (determined at visit 1 immediately following deployment) were included as covariates in regression models to adjust for differences between participants in traumatic exposure during and after combat, respectively (as detailed in the Methods section). Moreover, because the 4 MRS battalions differed from one another in their war zone experiences and in the timing of their training regimen relative to the period of data collection, cohort assignment of each participant was set as a factor in regression analysis. Multiple other potential confounders were evaluated, including several previously associated with both PTSD and peripheral inflammation (eg, baseline depression, anxiety, and alcohol and tobacco use[36-52]) and various anthropometric and demographic variables (Table 1); however, none met the criteria for inclusion in the regression models.

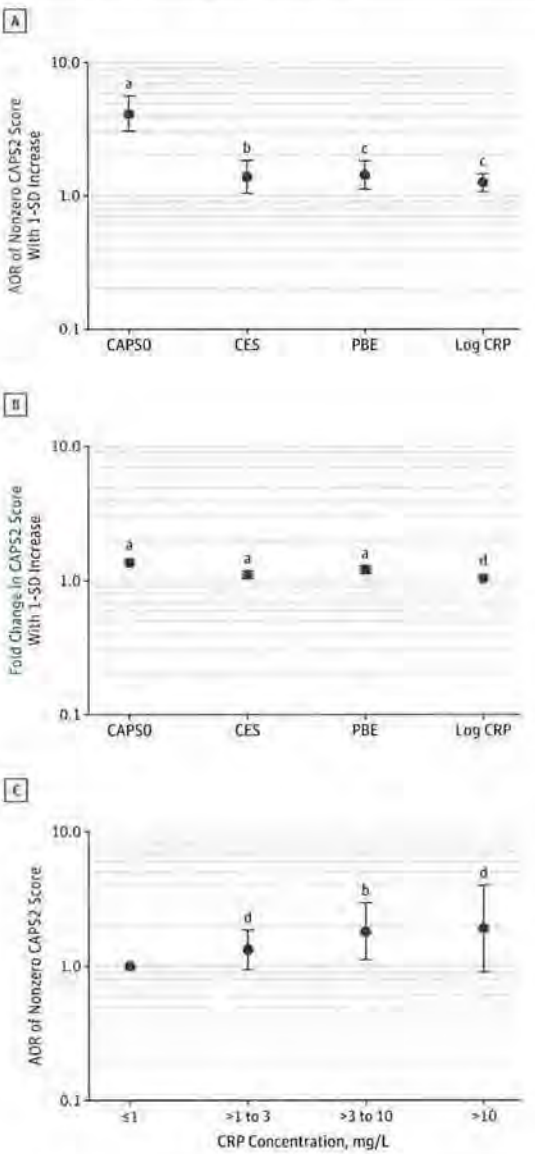
ZINBR of Postdeployment CAPS

In accordance with the analyses described above, our ZINBR model (described in the Methods section) of CAPS2 comprised plasma CRP concentration, CAPS0, cohort assignment, and CES and PBE scores. C-reactive protein was a highly significant overall predictor of CAPS2 in this model ($P = .002$ by likelihood ratio test), as was each of the other predictors (Table 2). C-reactive protein was also a highly significant predictor in the analogous linear regression model with the same covariates ($P = .002$); however, ZINBR is significantly superior to linear regression when modeling the outcome of CAPS (Supplement [eMethods]). We assessed all 2-way interactions with CRP; none was statistically significant. Based on analysis of the scores on CAPS subscales, the greatest effect of CRP appeared to be in the domain of hyperarousal (subscale D of CAPS; overall $P < .001$), with less of an effect on numbing (subscale CN; $P = .03$), and even lesser effects on reexperiencing (subscale B; $P = .28$) and avoidance (subscale CA; $P = .57$).

In the zero component of the ZINBR model, CRP was a positive predictor of CAPS2: each 1-U increment in $\log_{10}$ plasma CRP concentration (ie, each 10-fold increase in CRP concentration) was associated with a fold change in the approximate odds of obtaining a CAPS2 score greater than zero (ie, odds ratio [OR] of nonzero CAPS2) of 1.51 (95% CI, 1.15-1.97; $P = .003$) (Table 2). Stated in the context of the range of CRP concentrations in our study population, a 1-SD increase in $\log_{10}$ CRP (corresponding to a 3.57-fold increase in CRP concentration) was associated with an OR of 1.25 (1.08-1.45) (**Figure 1A**). By comparison, 1-SD increases in CAPS0, CES, and PBE were associated with ORs of 4.15 (95% CI, 3.06-5.63; $P < .001$), 1.39 (1.06-1.83; $P = .019$), and 1.43 (1.13-1.80; $P = .003$), respectively. Consistent with the findings obtained with CRP treated as a continuous predictor, categorization of CRP revealed a trend toward a greater OR of nonzero outcome with increasing CRP category (although there was, as expected, a loss of statistical power) (Figure 1B).

Likewise, CRP was a positive predictor of CAPS2 scores in the count component of the ZINBR model (which predicts approximately the extent of the outcome when it is nonzero, as described in the Supplement [eMethods]): each 10-fold increase in CRP concentration was associated with a 1.06-fold increase in CAPS2. However, this effect was statistically significant only at the trend level (95% CI, 0.99-1.14; $P = .09$

Figure 1. Effects of Baseline Plasma C-Reactive Protein (CRP) Concentration and Other Predictors on Postdeployment Posttraumatic Stress Disorder (PTSD) Symptoms



A, Adjusted odds ratios (AORs) of a nonzero Clinician-Administered PTSD Scale score at visit 2 (CAPS2) associated with 1-SD increases in the indicated variables. B, Adjusted fold changes in CAPS2 associated with 1-SD increases in the indicated variables. Data in A and B are from the zero-inflated negative binomial regression (ZINBR) model summarized in Table 2. C, The AORs of nonzero CAPS2 by baseline plasma CRP concentration category (reference category, ≤1; to convert to nanomoles per liter, multiply by 9.524), as determined by ZINBR and adjusted for the same covariates as the model in Table 2. CAPS0 indicates Clinician-Administered PTSD Scale at visit 0 (baseline); CES, Combat Exposure Scale; and PBE, Post-battle Experiences. Error bars delineate 95% CIs; the y-axes use a log scale. All *P* values are 2-tailed.

[a] $P < .001$.

[b] $P < .05$.

[c] $P < .01$.

[d] $P < .10$.

Table 3. Binomial Logistic Regression of Postdeployment (Visit 2) PTSD Diagnosis in 1719 Participants

Variable	OR (95% CI)[a,b]	*P* Value
Intercept[b]	0.01 (0.004-0.02)	<.001
Cohort 1	1.00 (0.41-2.45)	>.99
Cohort 2	1.13 (0.54-2.37)	.75
Cohort 3	0.81 (0.45-1.46)	.48
Cohort 4	0[c]	
CAPS0 score	1.06 (1.04-1.07)	<.001
CES score	1.03 (1.01-1.06)	.01
PBE score	1.06 (0.99-1.14)	.08
Log CRP	1.50 (1.02-2.22)	.04

Abbreviations: CAPS0, Clinician-Administered Posttraumatic Stress Disorder (PTSD) Scale score at visit 0 (baseline); CES, Combat Exposure Scale; CRP, baseline plasma C-reactive protein; OR, odds ratio; PBE, Post-battle Experiences.

[a] Odds ratio of PTSD (computed by exponentiating the corresponding coefficient in the regression model and adjusted for the variables listed in the table).

[b] Value for intercept indicates the odds of PTSD at baseline (cohort equals 4 and all other variables have zero values).

[c] This variable was set to zero because it is redundant.

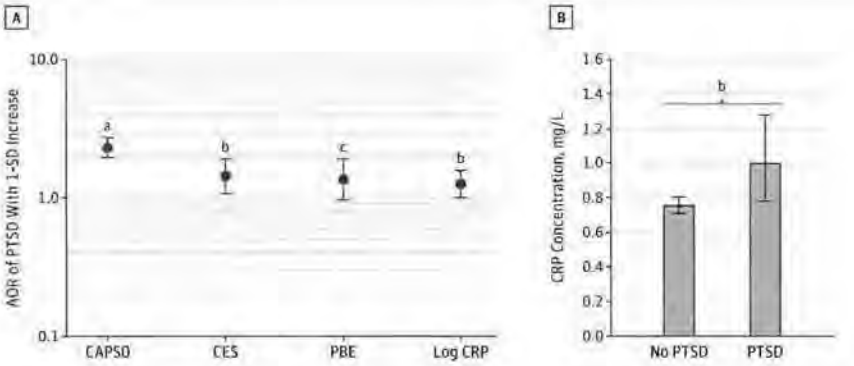
[2-tailed]) (Table 2). Accounting for the ranges in values of the predictors, a 1-SD increase in $\log_{10}$ CRP was associated with 1.03-fold change (95% CI, 1.00-1.07) in CAPS2; 1-SD increases in CAPS0, CES, and PBE were associated with fold changes of 1.36 (1.31-1.42; $P < .001$), 1.11 (1.05-1.18; $P = .001$), and 1.20 (1.13-1.28; $P < .001$), respectively (Figure 1B).

Logistic Regression of Postdeployment PTSD

In addition to analysis of the continuous outcome of CAPS2, we performed logistic regression of the categorical outcome of PTSD (*DSM-IV-R* definition [31]) at visit 2 using the same covariates as in the ZINBR model. C-reactive protein was a significant predictor in this analysis as well, albeit not to as great a degree as in the ZINBR model: each 10-fold increment in CRP concentration was associated with a PTSD OR of 1.50 (95% CI, 1.02-2.22; $P = .04$) (**Table 3**). Taking into account the ranges of predictor values, a 1-SD increase in $\log_{10}$ CRP was associated with a PTSD OR of 1.25; by comparison, 1-SD increases in CAPS0, CES, and PBE were associated with ORs of 2.30, 1.43, and 1.35, respectively (**Figure 2A**). Conversely, adjusted baseline CRP values for participants with and without PTSD at visit 2 were 1.00 and 0.76 mg/L (to convert to nanomoles per liter, multiply by 9.524), respectively (adjusted for the same covariates as the logistic regression model) (Figure 2B). C-reactive protein was similarly a significant predictor in ordinal logistic regression of the diagnostic categories of PTSD, partial PTSD,[32-34] or neither ($P = .03$) (Supplement [eResults, eTable, and eFigure]).

We also performed subgroup analyses excluding participants at various thresholds of the model variables: plasma CRP, baseline CAPS, CES, and PBE. C-reactive protein effects in these subsets were generally similar to those obtained when considering all participants, indicating that the effects are not substantially influenced by individuals at the extremes of plasma CRP, baseline PTSD symptoms, or combat exposure (data not shown). Moreover, CRP was not significantly associated with

Figure 2. Effects of Baseline Plasma C-Reactive Protein (CRP) Concentration and Other Predictors on Postdeployment Posttraumatic Stress Disorder (PTSD) Diagnosis



A, Adjusted odds ratios (AORs) of posttraumatic stress disorder (PTSD) at visit 2 associated with 1-SD increases in the indicated variables. Data are from the binomial logistic regression model summarized in Table 3. The y-axis uses a log scale. B, Baseline plasma CRP concentration of participants without or with PTSD at visit 2, adjusted for the same covariates as the logistic regression model in Table 3. CAPSO indicates Clinician-Administered PTSD Scale at visit 0 (baseline); CES, Combat Exposure Scale; PBE, Post-battle Experiences. Error bars delineate 95% CIs. All *P* values are 2-tailed.

[a] $P < .001$.

[b] $P < .05$.

[c] $P < .10$.

baseline CAPS or PTSD diagnosis ($P = .52$ and .22, respectively), indicating that CRP is not a mediator or proxy for the effects of one of these other predictors on CAPS2.

Discussion

We report a significant effect of baseline CRP on postdeployment PTSD symptom emergence in Marine and Navy combatants, suggesting that higher levels of this inflammatory marker may be prospectively associated with risk for PTSD. C-reactive protein predominantly influenced the likelihood of participants demonstrating the presence vs absence of PTSD symptoms rather than the extent of symptoms when present (as indicated by its greater statistical significance in the zero model of the ZINBR vs the count and logistic regression models) and had a greater effect on the hyperarousal and numbing symptom clusters than on the other clusters. Conceivably, high CRP levels mark a state of vulnerability to developing these symptoms of PTSD, and the influences of other factors prevail in determining the severity of symptoms once they are manifested.

It is sometimes postulated[7,25-27] that the observed association between PTSD and peripheral inflammation is due to the former disorder predisposing to the latter, plausibly due to PTSD-induced dysregulation of the stress axis resulting in disinhibition of proinflammatory pathways. Our data raise the converse possibility—that individuals with lesser inflammation may be relatively resilient and those with greater inflammation relatively vulnerable to developing PTSD symptoms. This supposition is also supported by the recent finding that the risk for PTSD following medical illness during military deployment is comparable to that following physical injury.[53,54] However, the possibility that higher CRP levels at baseline resulted from preceding trauma cannot be excluded.

The underlying mechanism may involve the actions of inflammatory cytokines, which, in addition to their well-characterized adverse effects on metabolic and therefore cardiovascular health,[12-14] have adverse effects on mental health.[55-57] In particular, depression has long been known to be associated with increased peripheral inflammation,[36,46-50] with some studies[37,38] suggesting that baseline inflammation may predict subsequent depression, and inflammatory cytokines are known to elicit symptoms of depression,[58-63] as discussed in reviews.[2,56,57,64] Furthermore, peripheral inflammation has been associated[65-69] with impairments in memory and executive function. Notably, inflammatory cytokines have been demonstrated to significantly suppress hippocampal neurogenesis in animals[70] and have been associated with low hippocampal volume in humans,[71] a neuroanatomical trait that might mark vulnerability to PTSD—in studies of identical twins discordant for combat trauma exposure, twin pairs in which the combat-exposed member developed PTSD had smaller hippocampi than the other twin pairs.[72,73]

Nevertheless, the causal relationships between psychiatric disorders and inflammation are likely to be complex. For instance, in one recent large, prospective, population-based study, cumulative episodes of depression predicted subsequent CRP levels[74] (although this effect was attenuated after controlling for body mass index and smoking, suggesting that it might be attributable in part to depression-related lifestyle changes rather than directly to the neurophysiological characteristics of depression). Moreover, with respect to PTSD, much work in animal models[75-77] supports the conclusion that chronic stress induces immunologic changes that culminate in a proinflammatory phenotype. Thus, inflammation may both contribute to PTSD and be a consequence of the stressors that led to the disorder.

Strengths of our study include its size, prospective design, and adjustment for multiple potential confounders.

Moreover, owing to the youth of the study participants (mean age, 22.78 years) and their relative physical fitness (given the requirements for combat deployment), it is unlikely that chronic physical illness confounded the observed effects of baseline CRP on postdeployment PTSD symptoms. However, certain limitations merit discussion. The relative fitness of our cohort also limits the generalizability of our findings, as does the absence of women. In addition, whereas CRP concentrations fluctuate substantially in response to transient inflammatory states (eg, minor infections[78]), values in our participants were determined only once and thus may be relatively "noisy." Moreover, use of anti-inflammatory medications, which might also have contributed to variability in CRP levels, was not ascertained in our study. However, such variability would generally be expected to bias toward the null hypothesis.

Finally, with regard to missing data, 27.2% of the participants did not have determination of CAPS2 scores. However, CRP values did not differ significantly between individuals for whom CAPS2 scores were present vs absent; conversely, CAPS2 scores were not significantly different when comparing participants for whom CRP values were present vs absent (not shown). Moreover, we found the effect size of CRP on CAPS2 or PTSD diagnosis (the CRP-associated OR or fold change) to be generally similar across subsets of participants having markedly different mean values for the various covariates in our regression models (data not shown). This suggests that even if the individuals with missing data were considerably different from the other participants with regard to CES, PBE, or baseline CAPS scores, the CRP effect sizes that would have been obtained had the data not been missing are likely to be similar to those that were in fact observed. Taken together, these results suggest that missing data might not have appreciably biased our findings concerning CRP effects on PTSD symptoms.

Conclusions

Our results, if validated by future studies, could have important clinical implications. If peripheral inflammation contributes to the development of PTSD, interventions to decrease inflammation, such as dietary or lifestyle modifications,[79-81] might ameliorate the severity of this disorder. At minimum, our findings are consistent with the adage *mens sana in corpore sano*: a healthy mind in a healthy body.

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RAPID PUBLICATION

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Blood-Based Gene-Expression Predictors of PTSD Risk and Resilience Among Deployed Marines: A Pilot Study

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Susceptibility to PTSD is determined by both genes and environment. Similarly, gene-expression levels in peripheral blood are influenced by both genes and environment, and expression levels of many genes show good correspondence between peripheral blood and brain. Therefore, our objectives were to test the following hypotheses: (1) pre-trauma expression levels of a gene subset (particularly immune-system genes) in peripheral blood would differ between trauma-exposed Marines who later developed PTSD and those who did not; (2) a predictive biomarker panel of the eventual emergence of PTSD among high-risk individuals could be developed based on gene expression in

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readily assessable peripheral blood cells; and (3) a predictive panel based on expression of individual exons would surpass the accuracy of a model based on expression of full-length gene transcripts. Gene-expression levels were assayed in peripheral blood samples from 50 U.S. Marines (25 eventual PTSD cases and 25 non-PTSD comparison subjects) prior to their deployment overseas to war-zones in Iraq or Afghanistan. The panel of biomarkers dysregulated in peripheral blood cells of eventual PTSD cases prior to deployment was significantly enriched for immune genes, achieved 70% prediction accuracy in an independent sample based on the expression of 23 full-length transcripts, and attained 80% accuracy in an independent sample based on the expression of one exon from each of five genes. If the observed profiles of pre-deployment mRNA-expression in eventual PTSD cases can be further refined and replicated, they could suggest avenues for early intervention and prevention among individuals at high risk for trauma exposure. © 2013 Wiley Periodicals, Inc.

Key words: alternative splicing; mRNA; peripheral blood mononuclear cells; transcriptome; trauma

INTRODUCTION

Previous research on post-traumatic stress disorder (PTSD) has identified numerous factors that put individuals at greater risk of developing the disorder, such as family history, childhood or early adulthood experiences, personality and cognitive traits, and pre-existing mental disorders [Koenen et al., 2005; Kremen et al., 2007]; however, no easily assessed biological markers of PTSD have yet been validated. The biological factors associated with the risk for (and resilience to) PTSD are also poorly understood. Although susceptibility to PTSD appears to be moderately heritable, non-genetic factors (most prominently the type and extent of the precipitating trauma, and social support) and gene–environment interactions likely also contribute to each individual's overall susceptibility to the disorder [True et al., 1993; Stein et al., 2002; Kremen et al., 2012].

Given the less-than-absolute heritability of PTSD, pursuit of genetic markers alone (e.g., single nucleotide polymorphisms and copy-number variations) will leave much of the variance in vulnerability unexplained [Yehuda et al., 2011; Mehta and Binder, 2012]. Gene expression (i.e., mRNA) levels, which potentially reflect the effects of both heredity and environment, may be better indicators of the aberrant biology underlying PTSD, as well as its premorbid risk state. PTSD clearly is a brain disorder, but assaying gene-expression levels—either acutely or longitudinally—in the brains of living human subjects at risk for PTSD is impossible. Yet, as demonstrated by Sullivan et al. [2006] and, more recently, Rollins et al. [2010] and Kohane and Valtchinov [2012], peripheral blood expression levels of many genes are moderately correlated with the expression levels of those genes in other tissues, including *postmortem* brain, suggesting the possibility that peripheral blood gene expression can be harnessed to construct useful profiles of brain disorders [Woelk et al., 2011]. Indeed, we and others have capitalized on this proxy phenomenon to identify promising peripheral blood-based biomarkers for a number of neuropsychiatric disorders, including schizophrenia, bipolar disorder, and autism spectrum disorders [Glatt et al., 2005, 2009, 2011a,b, 2012; Tsuang et al., 2005; Lee et al., 2012].

In the context of PTSD, several prior studies identified differences in peripheral blood gene-expression levels between individuals with PTSD and similarly exposed comparison subjects without PTSD. First, Segman et al. [2005] described a longitudinal analysis of gene expression in peripheral blood mononuclear cells (PBMCs) from trauma survivors at the emergency room immediately after their trauma and again 4 months later when a diagnosis of PTSD could be definitively established. Predictably, this study found that the expression of many genes previously implicated in mediating the stress response (e.g., genes associated with hypothalamic–pituitary–adrenal [HPA] axis function) were significantly dysregulated in subjects with PTSD relative to those who fully recovered from their trauma. These changes in gene expression also showed a linear relationship with the severity of three different clusters of PTSD symptoms. In addition to changes in stress-response genes, the PBMCs from subjects with full persistent PTSD were marked by significant down-regulation of transcriptional activators, suggesting that subjects with PTSD may experience a global deficiency in the production of mRNAs (and, thus, proteins) of key genes at critical times. Subsequently, Zieker et al. [2007] replicated dysregulation of stress-response genes in whole blood from a sample of subjects with long-persistent PTSD resulting from the same environmental trigger (the Ramstein air show catastrophe, 1989). In addition, Zieker et al., extended earlier work by demonstrating changes in several immune-related genes among PTSD sufferers. In 2009, Yehuda et al. [2009] identified a profile of dysregulated genes in peripheral blood of survivors of the World Trade Center attacks that also was enriched with genes involved in HPA axis and immune cell functions. Most recently, Neylan et al. [2011] found global down-regulation of genes in CD14+ monocytes from male PTSD sufferers, but some evidence of increased activation of immune-system genes in female PTSD patients.

Consolidating this evidence with the results from epidemiologic, genomic, and neurobiological studies of the disorder [e.g., Uddin et al., 2010] led us to recently propose a theory of PTSD predicated on dysregulation of immune and inflammatory processes in general, and cellular immunity in particular [Baker et al., 2012b]. However, it was not clear from any of this work whether dysregulation of these processes occurs only in response to trauma exposure or if, in fact, gene-expression abnormalities in peripheral blood of individuals *exist "pre-trauma" and signal a heightened susceptibility* to developing the disorder once trauma is experienced. Recent work by van Zuiden et al. [2012] supports the assertion that pre-trauma disturbances in peripheral blood gene expression (at least in the realm of glucocorticoid signaling and regulation of cell-mediated immune and inflammatory processes) may predict post-trauma onset of PTSD and depressive symptoms.

We virtually never know about exposure to a traumatic event in advance, so the next best alternative in the pursuit of PTSD biomarkers has historically been studies of people who have recently experienced a trauma. But the critical limitation in such studies is that it is not possible to differentiate pre-existing risk factors from the consequences of trauma exposure or of develop-

ment of PTSD. In the context of this prior work, we report here the results of transcriptome-wide expression-profiling of peripheral blood samples from individuals at uniquely elevated risk of trauma exposure and development of PTSD: participants in the Marine Resiliency Study (MRS) *prior to their deployment* to active war zones in Iraq or Afghanistan, who were then followed longitudinally [Baker et al., 2012a]. The objectives of this pilot study were to evaluate the following hypotheses: (1) pre-trauma expression levels of some genes (particularly immune-system genes) in peripheral blood cells would differ between trauma-exposed Marines who later went on to develop PTSD and those who did not; (2) a readily assessable, predictive biomarker panel of the eventual emergence of PTSD among high-risk individuals could be developed based on gene expression levels in peripheral blood cells; and (3) a predictive panel based on the expression of individual exons would surpass the accuracy of a model based on the expression of full-length transcripts of genes. We interpret the results of these analyses in two contexts: (1) as a means of identifying biological functions, processes, pathways, and protein domains whose genomic dysregulation may indicate or influence susceptibility to the disorder; and (2) the construction of predictive or prognostic classifiers that might ultimately find use in assessing individual risk for PTSD and implementing preventive strategies in such populations.

METHODS

Ascertainment and Clinical Characterization of Subjects

The MRS is a prospective cohort study of factors predictive of PTSD among approximately 2,600 Marines in four battalions deployed to Iraq or Afghanistan. The research team conducted structured clinical interviews on Marine bases and collected blood samples and data at four time points: pre-deployment, and ~1-week, ~3-months, and ~6-months after returning from deployment (i.e., post-deployment). Measures collected, including those used in this study, have been described in detail previously [Baker et al., 2012a].

The principal exclusion criteria for both affected cases and unaffected comparison subjects for the present analyses were: (1) a pre-deployment PTSD Checklist (PCL) score >44; and/or (2) a pre-deployment diagnosis of PTSD based on the Clinician-Administered PTSD Scale (CAPS). In other words, no included subjects met either clinician- or self-rated thresholds for a diagnosis of PTSD at pre-deployment. Cases were identified as those subjects who were issued a CAPS-based PTSD diagnosis at ~3- and/or ~6-months post-deployment. Unaffected comparison subjects were identified as those subjects who, at no time, attained a PCL score >44 and who were not issued a CAPS-based PTSD diagnosis at any post-deployment interview. Among subjects who were included in the full MRS sample and assigned to case or comparison groups based on these criteria, we then selected for analysis 25 male PTSD cases and 25 male comparison subjects based on similar demographics, pre-deployment clinical characteristics, deployment history, and levels of exposure to putative traumas as determined from the Combat and Post-Battle Experiences subscales of the Deployment Risk and Resilience Inventory (DRRI). After performing quality-control checks on the microarray data (described below), two subjects (one case and one comparison subject) were removed from analyses. The demographic, clinical, and combat-experiential characteristics of the remaining 24 case and 24 comparison subjects are shown in Table I. The two groups were comparable on all demographic and combat-experiential variables. Within both the case and comparison groups, 50% of the subjects had been deployed previously on at least one occasion, and while some subjects in each group had been previously deployed multiple times (up to three

TABLE I. Demographic, Clinical, and Experiential Characteristics of Eventual PTSD Cases and Non-PTSD Comparison Subjects

	Eventual PTSD cases	Comparison subjects	*P*-value
Sample size: *n*	24	24	
Age:	21.9 ± 3.2	21.5 ± 3.2	0.653
Previously deployed: *n* [%]	12 [50.0]	12 [50.0]	1.000
Ancestry: Caucasian *n* [%]	17 [70.8]	18 [75.0]	0.853
Cohort *n* [%]			
1	2 [8.3]	5 [20.8]	0.471
2	8 [33.3]	7 [29.2]	
3	14 [58.3]	12 [50.0]	
DRRI combat experiences	18.9 ± 13.1	20.2 ± 14.9	0.754
DRRI Post-battle experiences	7.3 ± 4.6	8.7 ± 4.0	0.281
CAPS pre-deployment	22.6 ± 12.0	15.4 ± 9.7	0.027
CAPS 3-months post-deployment	67.2 ± 21.8	40.0 ± 29.4	0.013
PCL Pre-Deployment	24.6 ± 6.4	23.2 ± 3.4	0.346
PCL 1-Week Post-Deployment	42.7 ± 17.6	23.0 ± 4.9	<0.001
PCL 3-months post-deployment	49.3 ± 12.5	21.2 ± ± 4.6	<0.001
PCL 6-months post-deployment	40.6 ± 13.8	20.1 ± 2.6	<0.001

Notes: (1) Demographic characteristics of each sample are reported as mean ± SD unless otherwise noted. (2) Sample means and proportions were compared using independent samples *t*-tests and chi-square tests, respectively.

times), there was no difference between the two groups in the proportion of multiply deployed individuals or in the average number of deployments. Although no subject met diagnostic threshold for PTSD at pre-deployment as determined by either clinician ratings on the CAPS or self-ratings on the PCL, the eventual PTSD cases did have significantly higher clinician ratings on the CAPS at pre-deployment, whereas no significant difference in pre-deployment self-ratings on the PCL were observed. As expected, the eventual PTSD cases also had significantly higher clinician- and self-rated symptoms of PTSD at all post-deployment evaluations.

mRNA Sample Acquisition, Stabilization, Isolation, and Storage

Close collaboration with the Marine Corps and the Navy, which provides health support for the Marine Corps, enabled comprehensive on-site data collection. The clinical interview and sample blood draw (10 ml) were both collected within 4 hr of each other on the same day. Each blood sample was collected into an EDTA-coated collection tube and immediately transferred to an RNase-free laboratory, where all subsequent procedures took place. The blood sample was passed over a LeukoLOCK filter, which was flushed with PBS and then fully saturated with RNAlater [Gonzales et al., 2005]. Each LeukoLOCK filter, containing bound, isolated, stabilized, and purified white blood cells, was sealed and stored in a sterile box at −20°C. Once mRNA samples were acquired from all subjects, the entire batch of samples was processed to isolate mRNA. Eluted mRNA samples were stored at −80°C until transferred to the SUNY MicroArray Core (SUNYMAC, Syracuse, NY) Facility at SUNY Upstate Medical University for quality assurance and microarray hybridization. LeukoLOCK filters, RNAlater, and TRI reagent were obtained from Applied Biosystems, Inc. (Foster City, CA), while all other reagents and supplies were obtained from VWR International, LLC (West Chester, PA) unless otherwise specified.

mRNA Quantitation, Quality Control, and Hybridization

The concentration of mRNA in each DNA-free sample was quantified by the absorption of ultraviolet light at two wavelengths (260 and 280 nm), which was measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific; Wilmington, DE). The quantity of mRNA in each of the 50 samples far exceeded the minimally sufficient amount required for microarray hybridization. The purity of each mRNA sample was estimated by the 260:280 nm absorbance ratio, with an acceptable range designated a priori as 1.7–2.1. The quality of each mRNA sample was quantified by the RNA Integrity Number (RIN) [Schroeder et al., 2006], which was determined on an RNA 6000 Labchip Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). According to convention [Schroeder et al., 2006], a RIN of 6.0 or greater was deemed to be indicative of acceptable quality, and no samples were removed based on this criterion. Two batches of 25 samples each (balanced with PTSD cases and controls) were then assayed on GeneChip Human Exon 1.0 ST Arrays (Affymetrix, Inc., Santa Clara, CA) per the "Whole Transcript Sense Target Labeling Assay" protocol [Affymetrix, 2006] using 1 μg of total RNA from each sample.

Microarray Data Import, Normalization, Transformation, Summarization, and Quality Control

Partek Genomics Suite software, version 6.6 © 2012 (Partek Incorporated, St. Louis, MO), was utilized for all analytic procedures performed on microarray scan data. Interrogating probes were imported, and corrections for background signal were applied using the robust multi-array average (RMA) method [Irizarry et al., 2003], with additional corrections applied for the GC-content of probes. The set of GeneChips was standardized using quantile normalization and expression levels of each probe underwent log-2 transformation to yield distributions of data that more closely approximated normality. As most genes were measured by multiple probe sets (typically one probe set per exon, but sometimes more), summarization of probes took place at two levels: first, probes tagging the same exon were summarized by median polish to arrive at one expression value per exon; second, exons tagging the same gene were summarized by median polish to arrive at one expression value per gene. All probesets were expressed with a signal:noise ratio ≥ 3; thus, no probesets were excluded from analyses of differential expression. A total of 257,106 probesets were analyzed, mapping to 20,224 whole transcripts and 209,826 exons.

Unsupervised clustering of subjects revealed no evidence of batch effects based on scan date. Principal components analysis (PCA) of the 50 pre-deployment data points identified two outliers (one case and one comparison subject) whose component values were beyond four standard deviations (SD) in each of the first three dimensions of the PCA plot, suggesting that the fundamental gene-expression pattern measured in these subjects (as evidenced by correlations among expression levels of probes) was inconsistent with that of the majority of other subjects. Both outlier samples exhibited high levels of average deviation among redundant probes located within a given chip, as well as high levels of average deviation in comparison with the median expression levels across all chips, suggesting either physical defects or hybridization problems with these chips. Removal of these two samples resulted in all 48 remaining subjects' data being well within the four-SD ellipsoid on each of the first three PCA dimensions.

Microarray Data Analyses

We performed four independent sets of analyses on the microarray data, as described below.

Identification of differentially expressed genes and their associated biological terms. We utilized analyses of covariance (ANCOVAs) to determine which full-length genetic transcripts were differentially expressed at pre-deployment in peripheral blood cells between PTSD cases and comparison subjects. We performed ANCOVAs of each gene's expression level as a function of PTSD status (case or control), deployment cohort (three levels corresponding to three platoons deployed at different times), age

(continuously measured in years), ancestry (dichotomized as Caucasian or not, as most subjects were Caucasian), and prior deployment status (first or subsequent deployment). Prior deployment accounted for less global variation in the expression dataset than did error, and prior deployment rates did not differ significantly between cases and comparison subjects, so it was removed from the model and subsequent analyses to preserve degrees of freedom.

To generate a relatively large candidate-gene list for functional profiling and construction of classifiers, we set the uncorrected type-I-error rate for diagnosis in these analyses at 0.01. We then reduced the dimensionality of the resulting list of candidate biomarkers through analysis of annotation-enrichment using the DAVID algorithm [Dennis et al., 2003] to determine if the gene list disproportionately represented any biological "terms." Specifically, we evaluated whether the list was enriched with genes that aggregated in the same functional categories, represented similar ontologies, participated in the same biological pathways, or exhibited common protein domains.The evaluated terms included: (1) ontologies from Gene Ontology Consortium (GOC) [Ashburner et al., 2000] and Clusters of Orthologous Groups (COG) [Tatusov et al., 2000]; (2) keywords from the Protein Information Resource (PIR) [Wu et al., 2003]; (3) features from the Universal Protein Resource (UniProt) [Apweiler et al., 2004]; (4) biological pathways from BioCarta and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [Kanehisa and Goto, 2000]; and (5) protein domains from PIR, the Integrative Protein Signature database (InterPro) [Hunter et al., 2009], the Simple Modular Architecture Research Tool (SMART) [Schultz et al., 1998], and the University of California at Santa Cruz's Transcription Factor Binding Site (TFBS) database. Bonferroni-correction was applied to the *P*-values obtained in the enrichment analyses of these annotation terms, and we only considered significant those tests that exceeded a threshold of $P = (0.05/\text{the number of terms evaluated in a particular category})$.

Discovery and replication of gene-based diagnostic predictors. We utilized a machine-learning technique (support vector machine, SVM) to construct, evaluate, optimize, and cross-validate classification algorithms predicting eventual PTSD status based on gene-expression levels at pre-deployment for a subset of our full sample. To accomplish this, we generated a large list of differentially expressed candidate genes (nominal $P < 0.01$) in a subset of the sample (19 cases and 19 comparison subjects) using ANCOVA and the same panel of factors and covariates described above. The probes on this list were then supplied as potential predictors in an SVM, as various model parameters and predictor combinations were evaluated to identify the model with the highest accuracy in identifying cases and comparison subjects based solely on the expression levels of a minimal gene set identified by shrinking centroids after two-level nested (i.e., two-level) 10-fold cross-validation. The top-performing model was then deployed on a fully independent test sample (five cases and five comparison subjects) to determine its generalizability in accurately predicting case status based on gene-expression levels (the 10 subjects used for model validation were not significantly different from those in the training set in terms of demographic, gene-expression QC, experiential, or clinical factors; data not shown).

Identification of differentially expressed exons and their associated biological terms. We examined exon-expression levels utilizing ANCOVAs to identify putative alternative splicing differences between individuals who would go on to develop PTSD and those who would not. The same factors evaluated in gene-based analyses (PTSD status, cohort, age, and ancestry) were assessed for their main effects and their interaction with exon ID as predictors of exon-expression levels, c.f. [Glatt et al., 2009]; however, due to the stronger effects of diagnosis on exon-specific expression observed relative to the earlier gene-based analyses, we restricted the candidate-gene list to transcripts with $P < 0.0001$ for the interaction of diagnosis and exon ID. This yielded a gene list still sufficiently large for the construction of classifiers (see below) and enrichment analyses, which we again performed using the DAVID algorithm. Enrichments were evaluated against a Bonferroni-corrected *P*-value accounting for the number of terms evaluated.

Discovery and replication of exon-based diagnostic predictors. As outlined above for full-length transcripts under Methods Section, we used SVMs to construct, evaluate, optimize, and cross-validate classification algorithms predicting eventual PTSD status based on exon-expression levels at pre-deployment for the same subset of our full sample. We first generated a large candidate list of putatively alternatively spliced genes (nominal $P < 0.0001$ for the interaction of PTSD status and exon ID) in a subset of the sample (19 cases and 19 comparison subjects) using ANCOVA and the same panel of factors, covariates, and interaction terms described above. For each gene on the list, the most significantly dysregulated exon was identified and supplied as a potential predictor in the SVM classifiers. Various model parameters and predictor combinations then were evaluated to identify the model with the highest accuracy in identifying cases and comparison subjects based solely on the expression levels of a minimal exon set identified by shrinking centroids after two-level nested 10-fold cross-validation. The top-performing model was then deployed on the fully independent test sample (five cases and five comparison subjects) to determine its generalizability in accurately predicting case status based on exon-expression levels.

RESULTS

Identification of Differentially Expressed Genes and Their Associated Biological Terms

No gene's expression level was related to future PTSD status at a Bonferroni-corrected level of significance, which is not surprising given the relatively small sample size and large number of transcripts tested. We did, however, identify 67 probes dysregulated with a nominally significant $P < 0.01$ in Marines who were later diagnosed with PTSD (Table II). Thirty-nine of these 67 probes were down-regulated, whereas 28 were up-regulated. While the direction of this pattern is consistent with prior work identifying transcriptional down-regulation in PTSD [Segman et al., 2005; Neylan et al., 2011], the ratio of down-regulated to up-regulated probes was not significantly different from chance expectation (one-tailed sign-test, $P = 0.11$). Log 2 fold-change (FC) of these probes in eventual PTSD cases ranged from 1.8-fold down-regulation to 2.1-fold up-regulation. Annotations significantly enriched in the list of 59 genes tagged by the 67 dysregulated probes—after Bonferroni correction for the number of terms

TABLE II. Genes Significantly Dysregulated [$P < 0.01$] in Peripheral Blood Mononuclear Cells from the Full Sample of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers

			Diagnostic group main effect		
Transcript cluster ID	Gene symbol	Gene product	Fold-change in cases	*F*	*P*-value
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	2.14	8.9	4.6E−03
7902541	*IFI44L*	Interferon-induced protein 44-like	1.77	7.8	7.9E−03
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3, 100 kDa	1.72	7.5	8.8E−03
7971296	*EPSTI1*	Epithelial stromal interaction 1 (breast)	1.68	11.7	1.4E−03
8050102	*CMPK2*	Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	1.54	9.2	4.1E−03
8071155	*USP18*	Ubiquitin specific peptidase 18	1.49	7.4	9.5E−03
7921434	*AIM2*	Absent in melanoma 2	1.46	8.8	4.9E−03
8046124	*DHRS9*	Dehydrogenase	1.44	8.9	4.7E−03
7958884	*OAS1*	2′,5′-oligoadenylate synthetase 1, 40	1.39	10.6	2.2E−03
7958913	*OAS2*	2′,5′-oligoadenylate synthetase 2, 69	1.38	8.5	5.7E−03
8004184	*XAF1*	XIAP associated factor 1	1.29	8.7	5.2E−03
7976443	*IFI27*	Interferon, alpha-inducible protein 27	1.26	11.6	1.4E−03
7953924	*CLEC9A*	C-type lectin domain family 9, member A	1.22	7.4	9.5E−03
8121532	*WISP3*	WNT1 inducible signaling pathway protein 3	1.22	8.6	5.3E−03
8107094	*ENST00000442824*	Cdna:pseudogene chromosome: GRCh37:5:97549106:97549825:	1.20	7.9	7.5E−03
8043375	*TRNK*	Mitochondrially encoded tRNA lysine	1.19	7.4	9.3E−03
8060294	*PDCD1*	Programmed cell death 1	1.18	8.2	6.5E−03
8127234	*DST*	Dystonin	1.15	9.0	4.4E−03
8018315	*SUMO2*	SMT3 suppressor of mif two 3 homolog 2 [*S. cerevisiae*]	1.14	9.9	2.9E−03
8060997	*SPTLC3*	Serine palmitoyltransferase, long chain base subunit 3	1.13	8.0	7.2E−03
8118345	*CFB*	Complement factor B	1.12	8.1	6.8E−03
8162884	*ALDOB*	Aldolase B, fructose-bisphosphate	1.11	10.5	2.3E−03
8061847	*C20orf70*	Chromosome 20 open reading frame 70	1.11	8.5	5.6E−03
8178115	*CFB*	Complement factor B	1.11	9.9	3.0E−03
7963386	*KRT82*	Keratin 82	1.10	9.2	4.1E−03
7990391	*CYP1A1*	Cytochrome P450, family 1, subfamily A, polypeptide 1	1.09	10.0	2.9E−03
8069503	*LOC441956*	Similar to cDNA sequence BC021523	1.09	19.0	8.0E−05
8139721	*ENST00000462919*	ncrna pseudogene:miRNA pseudogene chromosome: GRCh37:7:55713765:55713874	1.07	7.5	9.0E−03
7993146	*ENST00000475032*	ncrna pseudogene:scRNA pseudogene chromosome: GRCh37:16:8777112:8777408	−1.05	8.2	6.4E−03
8027824	*MAG*	Myelin associated glycoprotein	−1.08	7.6	8.5E−03
8142685	*TMEM229A*	Transmembrane protein 229A	−1.08	8.8	4.9E−03
8065252	*ENST00000432334*	cdna:known chromosome: GRCh37:20:19738352:19780320	−1.08	8.6	5.4E−03
8030002	*ZNF114*	Zinc finger protein 114	−1.09	9.6	3.4E−03
8118455	*C4A*	Complement component 4A (Rodgers blood group)	−1.09	9.1	4.3E−03
7945498	*SCT*	Secretin	−1.09	11.6	1.4E−03
8179399	*C4A*	Complement component 4A (Rodgers blood group)	−1.09	7.7	8.3E−03
8100523	*SPINK2*	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	−1.10	8.4	5.8E−03
8152812	*FAM84B*	Family with sequence similarity 84, member B	−1.10	10.3	2.6E−03
8024816	*FSD1*	Fibronectin type III and SPRY domain containing 1	−1.11	9.3	4.0E−03
8137962	*LOC100129484*	Hypothetical LOC100129484	−1.11	9.2	4.1E−03
8060339	*NRSN2*	Neurensin 2	−1.12	9.6	3.5E−03
7920264	*S100A5*	S100 calcium binding protein A5	−1.13	7.9	7.3E−03
8018646	*FOXJ1*	Forkhead box J1	−1.14	10.5	2.3E−03
8051061	*UCN*	Urocortin	−1.14	9.4	3.8E−03
8129095	*ENST00000435100*	Cdna:pseudogene chromosome: GRCh37:6:116579656:116580278	−1.15	7.6	8.7E−03
8122699	*RPS18P9*	Ribosomal protein S18 pseudogene 9	−1.15	8.9	4.6E−03

TABLE II. (*Continued*)

Transcript cluster ID	Gene symbol	Gene product	Diagnostic group main effect: Fold-change in cases	*F*	*P*-value
8012891	*ENST00000412454*	Cdna:pseudogene chromosome: GRCh37:17:14608393:14608851	−1.15	8.4	5.8E−03
8071368	*TMEM191A*	Transmembrane protein 191A	−1.15	8.9	4.7E−03
8127526	*RPL39*	Ribosomal protein L39	−1.15	8.0	7.2E−03
7985192	*AGPHD1*	Aminoglycoside phosphotransferase domain containing 1	−1.16	7.6	8.7E−03
8072584	*ENST00000042361*	Cdna:pseudogene chromosome: GRCh37:22:32435477:32435883	−1.16	9.4	3.7E−03
7992678	*LOC652276*	Hypothetical LOC652276	−1.16	7.4	9.5E−03
8118974	*RPL10A*	Ribosomal protein L10a	−1.17	9.3	3.9E−03
8147112	*CA13*	Carbonic anhydrase XIII	−1.17	8.1	6.7E−03
8063410	*PARD6B*	par-6 partitioning defective 6 homolog beta (C. elegans)	−1.17	8.5	5.5E−03
8148923	*LRRC14*	Leucine rich repeat containing 14	−1.18	7.8	7.8E−03
7953032	*LRTM2*	Leucine-rich repeats and transmembrane domains 2	−1.19	7.5	9.0E−03
8076260	*SLC25A17*	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34 kDa), member 17	−1.19	8.3	6.1E−03
7982271	*GOLGA8IP*	Golgin A8 family, member I (pseudogene)	−1.20	8.6	5.5E−03
7991742	*MPG*	*N*-methylpurine-DNA glycosylase	−1.20	9.2	4.1E−03
7905691	*RPS27*	Ribosomal protein S27	−1.20	7.7	8.0E−03
7950753	*CCDC90B*	Coiled-coil domain containing 90B	−1.24	10.2	2.6E−03
8103622	*CBR4*	Carbonyl reductase 4	−1.27	8.1	6.6E−03
8107520	*TNFAIP8*	Tumor necrosis factor, alpha-induced protein 8	−1.30	8.1	6.7E−03
7909601	*SNORA16B*	Small nucleolar RNA, H	−1.32	8.0	7.0E−03
8154962	*DNAJB5*	DnaJ (Hsp40) homolog, subfamily B, member 5	−1.35	10.4	2.4E−03
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	−1.83	10.2	2.7E−03

*Rows are sorted by decreasing fold-change in eventual PTSD cases relative to non-PTSD comparison subjects.

evaluated—included most prominently immune-related processes and protein domains involved in the response to viral infection (Table III), most of which were up-regulated in future PTSD cases. Exploratory pathway analysis of the differentially expressed genes in Table II using the Reactome database [Matthews et al., 2009] revealed that a subset of genes involved in type-1 interferon signaling represented the only significantly enriched pathway within our dataset. Six of the 59 genes were differentially expressed (*IFI27*, *OAS1*, *OAS2*, *OAS3*, *XAF1*, and *USP18*), with all probes up-regulated in future PTSD cases.

Discovery and Replication of a Gene-Based Diagnostic Predictor

To construct a gene-based classifier and assess its generalizability, we first derived a list of potential classifier transcripts as those probes with a difference in expression between PTSD case and comparison subjects attaining $P < 0.01$ in the training sample of 19 cases and 19 comparison subjects while controlling for the same factors and covariates as in analysis 1. This analysis and filtering left 61 probes (Table IV) that were then used to build and optimize SVM classifiers. The optimal SVM (identified through two-level nested 10-fold cross-validation with shrinking centroids, cost = 401, tolerance = 0.001, kernel = radial basis function, and gamma = 0.001) comprised 23 of the 61 starting probes (Table IV, probes in bold font) and attained 85% accuracy in classifying those individuals in the training sample who would or would not go on to develop PTSD. We then tested the identical 23-gene SVM (with the same parameters, but with no shrinkage or cross-validation) in the remaining independent test cohort (five cases and five comparison subjects), where it yielded a diminished but still reasonable 70% accuracy. Among cases, three of five were correctly classified, while four of five comparison subjects were classified correctly. These values correspond to a sensitivity, specificity, positive predictive value, and negative predictive value in the test sample of 60%, 80%, 75%, and 67%, respectively.

Identification of Differentially Expressed Exons and Their Associated Biological Terms

The interaction of diagnosis and exon ID identified putative isoform-expression differences ($P < 0.0001$) in 13 genes, seven of which attained Bonferroni-corrected significance (Table V). An example of between-group differences in exon expression for one of these five genes (*SUV420H1*) is illustrated in Figure 1, where the future PTSD cases have significantly lower levels of expression of a

TABLE III. Annotations Enriched at Bonferroni-Corrected Significant Levels Among Genes Dysregulated ($P < 0.01$) in Peripheral Blood Mononuclear Cells From the Full Sample of Eventual PTSD Cases at Pre-Deployment*

Category	Term	Count (%)	Fold enrichment	*P*-value	Bonferroni corrected *P*	Dysregulated genes (direction of dysregulation in eventual PTSD cases)
GOTERM_BP_FAT	GO:0006955 ~ immune response	10 (2.2)	5.6	3.7E−05	1.8E−02	*OAS1*(↑), *OAS2*(↑), *AIM2*(↑), *PDCD1*(↑)
INTERPRO	IPR006117:2′,5′-oligoadenylate synthetase, conserved site	3 (0.7)	277.7	4.1E−05	5.7E−03	*OAS3*(↑), *OAS1*(↑), *OAS2*(↑)
INTERPRO	IPR018952:2′,5′-oligoadenylate synthetase 1, domain 2/C-terminal	3 (0.7)	277.7	4.1E−05	5.7E−03	*OAS3*(↑), *OAS1*(↑), *OAS2*(↑)
INTERPRO	IPR006116:2′,5′-oligoadenylate synthetase, ubiquitin-like region	3 (0.7)	222.1	6.8E−05	9.5E−03	*OAS3*(↑), *OAS1*(↑), *OAS2*(↑)

*Rows are sorted by increasing *P*-value for the enrichment of annotations.

TABLE IV. Genes Significantly Dysregulated ($P < 0.01$) in Peripheral Blood Mononuclear Cells From a Subset of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers

			Diagnostic group main effect		
Transcript cluster ID[a]	Gene symbol	Gene product	Fold-change in cases	*F*	*P*-value
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	2.33	9.3	4.6E−03
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3, 100 kDa	2.01	11.0	2.3E−03
7902541	*IFI44L*	Interferon-induced protein 44-like	1.99	11.0	2.3E−03
8064716	*SIGLEC1*	Sialic acid binding Ig-like lectin 1, sialoadhesin	1.48	10.4	2.9E−03
7958913	*OAS2*	2′,5′-Oligoadenylate synthetase 2, 69	1.45	8.0	8.2E−03
8165682	*TRNS2*	Mitochondrially encoded tRNA serine 2	1.38	8.2	7.5E−03
8102127	*TACR3*	Tachykinin receptor 3	1.35	7.6	9.6E−03
7971191	*SUGT1P3*	Suppressor of G2 allele of SKP1 (S. cerevisiae) pseudogene 3	1.27	8.4	6.7E−03
8043375	*TRNK*	Mitochondrially encoded tRNA lysine	1.25	8.4	6.8E−03
8165684	*TRNL2*	Mitochondrially encoded tRNAleucine 2	1.25	8.6	6.2E−03
8165667	*TRNK*	Mitochondrially encoded tRNA lysine	1.25	8.1	7.5E−03
7896752	*TRNK*	Mitochondrially encoded tRNA lysine	1.25	8.1	7.5E−03
8055594	*ENSEMBL*	ncrna_pseudogene:Mt_tRNA_pseudogene chromosome: GRCh37:2:1	1.23	8.2	7.3E−03
7903203	*SNX7*	sorting nexin 7	1.21	9.4	4.4E−03
7938561	*ENST00000487144*	ncrna_pseudogene:rRNA_pseudogene chromosome: GRCh37:11:132	1.16	10.8	2.4E−03
8087433	*NICN1*	Nicolin 1	1.16	7.9	8.3E−03
8060997	*SPTLC3*	Serine palmitoyltransferase, long chain base subunit 3	1.15	9.0	5.1E−03
8031680	*ENST00000492903*	ncrna_pseudogene:Mt_tRNA_pseudogene chromosome: GRCh37:19:	1.13	8.3	7.1E−03
7953697	*GENSCAN00000020682*	cdna:Genscan chromosome: GRCh37:12:8090472:8168935:1	1.07	8.1	7.5E−03
8141423	*MIR106B*	microRNA 106b	−1.05	9.3	4.5E−03
8091099	*ENST00000450495*	cdna:known chromosome: GRCh37:3:141583849:141584121:-1 gen	−1.06	8.7	6.0E−03
8146643	*MIR124-2*	microRNA 124-2	−1.07	8.3	7.0E−03
8027824	*MAG*	Myelin associated glycoprotein	−1.08	8.2	7.3E−03
7911941	*CHD5*	Chromodomain helicase DNA binding protein 5	−1.08	8.3	6.9E−03
7955211	*DNAJC22*	DnaJ (Hsp40) homolog, subfamily C, member 22	−1.08	9.4	4.4E−03

TABLE IV. (*Continued*)

			Diagnostic group main effect		
Transcript cluster ID[a]	**Gene symbol**	**Gene product**	**Fold-change in cases**	***F***	***P*-value**
8055314	*LYPD1*	LY6	−1.09	7.6	9.7E−03
8065252	*BC004382*	*Homo sapiens*, clone IMAGE:3640982, mRNA, partial cds	−1.10	7.7	9.0E−03
8100523	*SPINK2*	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibi	−1.10	11.2	2.1E−03
8030002	*ZNF114*	Zinc finger protein 114	−1.11	15.2	4.6E−04
8060339	*NRSN2*	Neurensin 2	−1.11	13.1	9.9E−04
8152812	*FAM84B*	Family with sequence similarity 84, member B	−1.11	7.9	8.3E−03
8112072	*CCNO*	Cyclin O	−1.11	7.7	9.3E−03
7945498	*SCT*	Secretin	−1.12	14.8	5.3E−04
8126450	*RPL24*	Ribosomal protein L24	−1.13	7.5	9.8E−03
8084478	*FAM131A*	Family with sequence similarity 131, member A	−1.13	8.1	7.7E−03
8042532	*VAX2*	Ventral anterior homeobox 2	−1.13	17.5	2.1E−04
8151281	*TRAM1*	Translocation associated membrane protein 1	−1.13	8.4	6.7E−03
8019687	*ANAPC11*	Anaphase promoting complex subunit 11	−1.14	9.4	4.4E−03
8074869	*RTDR1*	Rhabdoid tumor deletion region gene 1	−1.14	8.1	7.8E−03
7928107	*H2AFY2*	H2A histone family, member Y2	−1.14	8.0	7.9E−03
8024816	*FSD1*	Fibronectin type III and SPRY domain containing 1	−1.15	12.2	1.4E−03
8038048	*CCDC114*	Coiled-coil domain containing 114	−1.15	9.3	4.5E−03
8084982	*LOC152217*	Hypothetical LOC152217	−1.15	7.6	9.7E−03
8127526	*RPL39*	Ribosomal protein L39	−1.17	8.8	5.6E−03
8154563	*ACER2*	Alkaline ceramidase 2	−1.18	9.9	3.5E−03
7985192	*AGPHD1*	Aminoglycoside phosphotransferase domain containing 1	−1.18	7.9	8.5E−03
8178090	*C6orf48*	Chromosome 6 open reading frame 48	−1.18	7.9	8.5E−03
8179326	*C6orf48*	Chromosome 6 open reading frame 48	−1.18	7.9	8.5E−03
8018646	*FOXJ1*	Forkhead box J1	−1.18	9.8	3.7E−03
8072584	*ENST00000423610*	cdna:pseudogene chromosome: GRCh37:22:32435477:32435883:1	−1.19	8.7	5.9E−03
8022170	*RPL6*	Ribosomal protein L6	−1.20	9.0	5.2E−03
7932964	*C1D*	C1D nuclear receptor corepressor	−1.21	8.9	5.4E−03
8085852	*NGLY1*	*N*-glycanase 1	−1.22	9.3	4.6E−03
8160308	*RPS6*	Ribosomal protein S6	−1.22	9.8	3.7E−03
8038993	*ZNF28*	Zinc finger protein 28	−1.25	8.4	6.8E−03
8107520	*TNFAIP8*	Tumor necrosis factor, alpha-induced protein 8	−1.29	7.9	8.5E−03
7911359	*NOC2L*	Nucleolar complex associated 2 homolog (S. cerevisiae)	−1.29	7.8	8.9E−03
8119357	*DAAM2*	Dishevelled associated activator of morphogenesis 2	−1.30	9.8	3.7E−03
8090256	*SNX4*	Sorting nexin 4	−1.37	8.8	5.6E−03
8155359	*CNTNAP3*	Contactin associated protein-like 3	−1.42	7.7	9.1E−03
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	−1.95	9.2	4.8E−03

[a]Rows are sorted by decreasing fold-change in eventual PTSD cases relative to non-PTSD comparison subjects.
[b]Transcripts in bold comprised the optimal 23-probe SVM classifier of eventual PTSD status identified by training and testing in independent samples.

single probe in the 3′ (left) end of the gene suggesting lower expression of the *b* isoform (one of the gene's 12 known isoforms) among future PTSD cases. The list of 13 genes was analyzed by the DAVID algorithm, but no annotations were found to be significantly enriched after Bonferroni correction for the number of terms evaluated; this is not surprising based on the small size of this gene list, which did not afford much opportunity for enrichment to be detected.

Discovery and Replication of an Exon-Based Diagnostic Predictor

To construct an exon-based classifier and assess its generalizability we first identified potentially differentially spliced exons within our training sample of 19 cases and 19 comparison subjects based on the diagnosis × exon ID interaction term, using a nominal threshold of $P < 0.0001$, while controlling for the same factors and covariates

TABLE V. Exons Significantly Dysregulated in Peripheral Blood Mononuclear Cells From the Full Sample of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers[a]

Transcript cluster ID	Gene symbol	Gene product	Accession number	*F*	*P*	Adjusted *P*	q	Probesets (n)	Dysregulated probeset IDs[b]
7954810	*LRRK2*	Leucine-rich repeat kinase 2	NM_198578	3.22	2.0E−13	4.1E−09	4.1E−09	53	7954813, 7954814, 7954818, **7954820**, 7954832, 7954833, 7954845, 7954854, 7954856, 7954863
8068740	*UMODL1*	Uromodulin-like 1	NM_001004416	5.28	3.4E−12	3.4E−08	6.9E−08	20	**8068745**, 8068747
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	NM_080657	7.94	8.5E−10	5.7E−06	1.7E−05	9	8040082, 8040083, 8040084, **8040085**, 8040086, 8040087, 8040088
7949931	*SUV420H1*	Suppressor of variegation 4-20 homolog 1 (Drosophila)	NM_017635	5.06	1.7E−08	8.6E−05	3.4E−04	14	**7949933**
8136662	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	NM_004668	2.38	1.0E−06	4.1E−03	0.02	46	—
8163535	*AMBP*	Alpha-1-microglobulin	NM_001633	4.18	6.6E−06	2.2E−02	0.13	12	8163538, **8163541**, 8163547
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	NM_000561	4.85	1.1E−05	3.3E−02	0.23	9	7903755, 7903767, 7903768, **7903769**, 7903771, 7903772, 7903773, 7903774
8128459	*SIM1*	Single-minded homolog 1 (Drosophila)	NM_005068	3.87	2.3E−05	0.06	0.46	12	8128464, **8128465**
8154962	*DNAJB5*	DnaJ (Hsp40) homolog, subfamily B, member 5	NM_001135004	4.22	3.2E−05	0.07	0.64	10	8154966, **8154967**, 8154968, 8154969
8051061	*UCN*	Urocortin	NM_003353	8.46	3.6E−05	0.07	0.73	4	**8051062**
8018315	*SUMO2*	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae)	NM_006937	4.80	3.9E−05	0.07	0.79	8	8018318, **8018319**
8107356	*DCP2*	DCP2 decapping enzyme homolog (S. cerevisiae)	NM_152624	3.12	6.0E−05	0.10	1.00	16	8107358, 8107359, **8107363**
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3	NM_006187	3.00	7.2E−05	1.00	1.00	17	7958898, 7958899, 7958901, 7958903, 7958904, 7958905, 7958907, 7958908, 7958910, 7958909, 7958911, **7958912**

[a]Rows are sorted by increasing *P*-value for the interaction of diagnosis and exon ID.
[b]Exon probesets in bold were the most significantly differentially expressed (per gene) between diagnostic groups, and were used in SVM classification analyses.

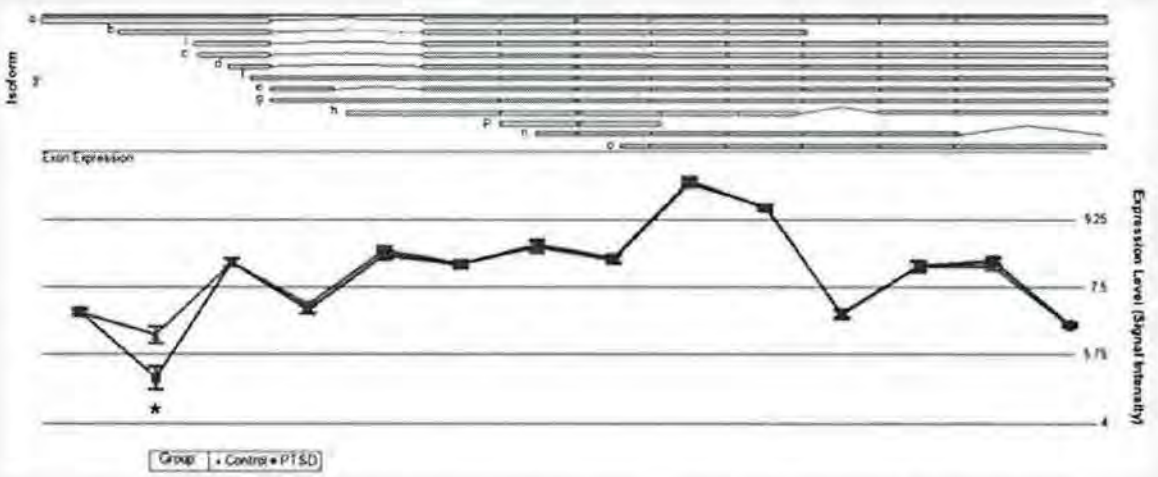

FIG. 1. Microarray-derived expression levels (ordinate) of individual exon-probes (abscissa) of suppressor of variegation 4–20 homolog 1 of *Drosophila* (*SUV420H1*) in peripheral blood mononuclear cells from eventual PTSD cases ($n = 24$; squares) and comparison subjects ($n = 24$; triangles). The interaction of diagnosis and exon ID was highly significant ($p = 1.7E-08$, Bonferroni-corrected $p = 3.4E-04$) owing to the selective down-regulation of an extended exon (probeset ID 7949933) in the 3′ end of isoform b ($*p = 0.005$) in eventual PTSD cases which occurs in the context of comparable expression levels of all other exons and isoform between groups.

as in the analyses above. For genes displaying more than one dysregulated probe between diagnostic groups, we selected the probe with the most significant between-group difference in expression level based on the *P*-values from planned comparisons. This analysis and filtering yielded 11 exons with expression differences between PTSD cases and comparison subjects (Table VI) that were then used to build and optimize SVM classifiers. The optimal SVM (identified through two-level nested 10-fold cross-validation with shrinking centroids, cost = 201, tolerance = 0.001, kernel = radial basis function, and gamma = 0.0001) comprised five of the 11 starting probes (Table VI, probes in bold font) and attained 84% accuracy in classifying those individuals in the training sample who would or would not go on to develop PTSD. We then tested the identical five-gene SVM (with the same parameters, but with no shrinkage or cross-validation) in the remaining independent test cohort (n = 10; five cases and five comparison subjects), where it yielded a diminished but reasonable 80% accuracy (higher than the accuracy observed in gene-based analyses). Among PTSD cases, three of five were correctly classified while all five comparison subjects were classified correctly. These values correspond to

TABLE VI. Exons Significantly Dysregulated in Peripheral Blood Mononuclear Cells From a Subset of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers[a]

Transcript cluster ID[b]	Gene symbol	Gene product	Interaction *P*	Exon ID	Fold-change	*F*	*P*-value
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	1.3E−07	8040085	2.46	10.42	2.9E−03
8133788	*PTPN12*	Protein tyrosine phosphatase, non-receptor type 12	1.8E−05	8133802	2.23	7.64	9.4E−03
8136662	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	4.8E−06	8136700	2.20	3.36	7.6E−02
8064716	*SIGLEC1*	Sialic acid binding Ig-like lectin 1, sialoadhesin	5.9E−06	8064717	1.80	15.42	4.3E−04
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3	2.7E−05	7958912	1.74	5.64	4.2E−03
7954810	*LRRK2*	Leucine-rich repeat kinase 2	8.1E−09	7954820	1.21	8.53	6.3E−03
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	7.4E−05	7903769	−1.48	8.31	7.0E−03
8107356	*DCP2*	Decapping enzyme homolog (S. cerevisiae)	9.7E−05	8107363	−2.04	8.29	7.1E−03
7949931	*SUV420H1*	Suppressor of variegation 4-20 homolog 1	5.1E−06	7949933	−2.21	6.13	1.9E−02
8083282	*HPS3*	Hermansky-Pudlak syndrome 3	2.9E−06	8083291	−2.28	6.18	1.8E−02
8068740	*UMODL1*	Uromodulin-like 1	2.7E−19	8068745	−7.13	16.93	2.5E−04

[a]Rows are sorted by decreasing fold-change in eventual PTSD cases relative to non-PTSD comparison subjects.
[b]Exons of transcript cluster IDs in bold comprised the optimal 5-probe SVM classifier of eventual PTSD status identified by training and testing in independent samples.

sensitivity, specificity, positive predictive, and negative predictive values of 60%, 100%, 100%, and 71%, respectively.

DISCUSSION

A fairly consistent picture of PTSD-induced or -associated changes in peripheral blood gene expression is emerging, with immunity-related genes among the most reliably implicated biomarkers. To this picture we add new and compelling pilot data suggesting that dysregulation of immunity-related genes not only accompanies the emergence of PTSD, but precedes it. This result strongly suggests that this dysregulation is a risk factor and not simply a consequence of PTSD. Yet, immune-gene dysregulation may be only one piece of the biological puzzle of PTSD susceptibility, as many genes comprising the best-performing PTSD-predictive classifiers were not immune-system genes, and these other genes had highly disparate functions.

Collectively, profiles of dysregulated genes in immune and other pathways may serve as potent risk indicators upon which early intervention and prevention efforts may ultimately be based. *To wit*, we were able to construct and validate two panels of blood-based PTSD risk-predictive biomarkers that ranged in accuracy from 70% to 80% in independent (albeit small) replication samples. Despite our relatively small sample size and the additional levels of correction for multiple-testing required for exon analyses, a number of differentially expressed exons surpassed stringent criteria for declaring statistical significance. Additionally, the exon-based predictive classifier appeared to perform better than the gene-based predictive classifier. Taken together, these findings suggest that exon expression may be more reliable and biologically informative than gene expression (which reflects the average expression of all transcript isoforms of a particular cluster).

It is important to note that these classifiers employed decision-rules based solely on mRNA expression levels. Possibly, more accurate classification models can be constructed in the future by taking into account additional known predictors of PTSD, such as family history, personality traits, pre-existing mental disorders [Koenen et al., 2003a,b], and other factors not necessarily related to gene expression. Alternatively, risk factors such as childhood exposure to trauma [van Zuiden et al., 2012] might actually be associated with or interact with alterations in pre-deployment mRNA-expression profiles. The present study was unable to account for childhood exposure to trauma or other such factors, but future efforts to construct predictive models should seek to incorporate such data. Further precision in measuring the amounts and types of mRNA isoforms present in peripheral blood (e.g., by further analyses of exon-level expression, or by quantitation of distinct alternatively spliced isoforms through RNAseq or exon–exon junction-probing microarrays) will undoubtedly also facilitate the construction of more accurate classifiers. Nevertheless, a single predictive classifier of PTSD (no matter how precisely constructed) may never perform with 100% accuracy, which is why it will be essential to pursue (in larger samples) those characteristics of either the subjects or the data that would determine for whom such a classifier works. Of equal interest is the possibility that, despite similar phenotypic manifestations of PTSD, there are two or more unique biomarker profiles that predict the same phenotypic outcome. In fact, etiologic heterogeneity may be a hallmark of complex disorders including PTSD, so it may not be possible to identify a single "one-size-fits-all" biomarker profile of the susceptibility toward the disorder. Thus, in the future, distinct predictive biomarker classifiers may be required to account for disorder stratification and correctly classify biologically or phenotypically separate sets of subjects at highest risk of developing PTSD. Another distinct possibility is that for some eventual cases of PTSD there is no blood-based pre-trauma biomarker signature of increased susceptibility to be found. We are currently investigating each of these possibilities further.

Because of our relatively small sample size and the severe corrections for multiple-testing required when examining the entire transcriptome, we did not detect individual gene-expression differences in eventual PTSD cases that surpassed stringent criteria for declaring statistical significance. As such, the focus of our efforts and interpretations has been on groups of genes, either in regard to their biological annotations or their collective ability to identify PTSD cases. Nevertheless, one gene identified here as predictive of PTSD emergence (*RPL24*) is notable in that it was also identified as a diagnostic biomarker of PTSD in a prior blood-based gene expression study by Mehta and Binder [2012]. Interestingly, we found that this gene was significantly down-regulated at pre-deployment among Marines who would later go on to develop PTSD, whereas Mehta et al., found this gene to be up-regulated in current PTSD sufferers. If this observation can be confirmed by additional work, it suggests that the down-regulation of *RPL24* at baseline may signal heightened susceptibility for the disorder which is then accompanied by a concomitant increase and over-expression of this gene after exposure to the precipitating trauma and subsequent development of PTSD symptoms. The majority of genes that we found to be dysregulated at baseline in eventual PTSD cases do not appear in other post-trauma studies to be either significantly up- or down-regulated in established PTSD cases, suggesting that the expression levels of these genes simply signify a risk state but do not necessarily bear on the presentation of the disorder once trauma has been experienced. Our results must be validated using another more sensitive mRNA-quantification technique such as qRT-PCR, but beyond this, replication in other well-powered longitudinal studies of subjects at high risk for trauma will prove crucial for more definitively implicating particular genes as risk indicators.

The present pilot study broadens the search for pre-deployment biomarkers for PTSD vulnerability beyond that of previous work [e.g., van Zuiden et al., 2012]. To our knowledge, this was the first study to search transcriptome-wide for patterns of gene- and exon-expression that distinguished future PTSD cases from non-PTSD comparison subjects. The present study is also unique because it employed a data-driven machine-learning approach for identifying the transcripts that, collectively, were most predictive of future PTSD status, many of which had not previously been associated with PTSD. Taken together, these two strategies are useful for identifying exons, genes, and pathways that potentially serve as biomarkers and play a role in the etiology of PTSD, but that may have been overlooked by other approaches focusing on well-established candidate genes.

This work must be considered in the context of its limitations. Foremost among these may be the observation of an increased pre-

deployment CAPS score among future PTSD cases. A closer examination of this finding revealed that this difference was driven by the "D" subscale of the CAPS measure, reflecting an increased reporting of symptoms of hyper-arousal among future cases. Because of this limitation, it cannot be determined unequivocally whether the present study has detected true biological vulnerability, pre-clinical changes associated with PTSD, or (more likely) some combination of these factors. Conclusions about the origins of the blood-based biomarker signals (vulnerability vs. preclinical state) could be strengthened in future studies by controlling for the severity of prior trauma exposure, or better yet, by examining pre-deployment gene expression in trauma-naïve subjects. Nevertheless, we maintain that the design of our study lends itself to the potential development of a predictive biomarker with some clinical utility; one that potentially can be used to determine who is at increased risk for emergent PTSD among a group of real-world service members who will undoubtedly have mixed and incomplete records of trauma exposure and may even manifest signs of pre-clinical disorder.

Regardless of the preliminary state of our conclusions regarding individual genes, our work makes clear that genes involved in cellular immunity are reliably and disproportionately represented among those that are dysregulated (mostly up-regulated) in our sample of eventual PTSD cases. This finding is consistent with evidence for dysfunctional cellular immune processes in individuals with PTSD, which we recently reviewed in depth [Baker et al., 2012b]. Our review of the collective evidence suggests that systemic inflammation and deleterious health consequences in PTSD are strongly linked. Given this evidence, treatment strategies to reduce inflammation that target biobehavioral factors may be of value to pursue.

In conclusion, as the development of PTSD following initial trauma exposure is quite variable and unpredictable, we sought to identify readily assessable biomarkers of risk and resilience based on evaluations of blood-based gene expression among soon-to-be-deployed Marines participating in the MRS. Our analyses converged on the immune-system as the most reliably dysregulated biological process characterizing high-risk individuals; however, numerous other genes not strictly related to cellular immunity also appear to be differentially expressed at baseline in individuals who develop PTSD, and these genes contribute much to our blood-based prediction models of the disorder's emergence. If biomarkers related to PTSD risk and resilience (such as the panels of genes and exons identified here) can be validated in additional cohorts and prospective studies, they may help to confidently identify which individuals are at the highest risk in real-world scenarios. These efforts may lead to more effective primary prevention protocols, which would be particularly important in groups such as these Marines for whom it is known in advance that exposure to serious trauma is highly likely. This may also prove highly relevant for first-responders, such as police, fire, and emergency medical teams, for whom a regular part of their job is also exposure to potentially traumatic situations. Further work correlating pre- and post-deployment differences in gene expression among PTSD cases and unaffected comparison subjects would also constitute a major advance in the effort to identify the biological mechanisms of this disorder and potentially develop diagnostic biomarkers that can serve as useful adjuncts to the prevailing gold-standard behavioral diagnostic systems [Brewin et al., 2000; Ozer et al., 2003].

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Single-subject-based whole-brain MEG slow-wave imaging approach for detecting abnormality in patients with mild traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is a leading cause of sustained impairment in military and civilian populations. However, mild TBI (mTBI) can be difficult to detect using conventional MRI or CT. Injured brain tissues in mTBI patients generate abnormal slow-waves (1–4 Hz) that can be measured and localized by resting-state magnetoencephalography (MEG). In this study, we develop a voxel-based whole-brain MEG slow-wave imaging approach for detecting abnormality in patients with mTBI on a single-subject basis. A normative database of resting-state MEG source magnitude images (1–4 Hz) from 79 healthy control subjects was established for all brain voxels. The high-resolution MEG source magnitude images were obtained by our recent Fast-VESTAL method. In 84 mTBI patients with persistent post-concussive symptoms (36 from blasts, and 48 from non-blast causes), our method detected abnormalities at the positive detection rates of 84.5%, 86.1%, and 83.3% for the combined (blast-induced plus with non-blast causes), blast, and non-blast mTBI groups, respectively. We found that prefrontal, posterior parietal, inferior temporal, hippocampus, and cerebella areas were particularly vulnerable to head trauma. The result also showed that MEG slow-wave generation in prefrontal areas positively correlated with personality change, trouble concentrating, affective lability, and depression symptoms. Discussion is provided regarding the neuronal mechanisms of MEG slow-wave generation due to deafferentation caused by axonal injury and/or blockages/limitations of cholinergic transmission in TBI. This study provides an effective way for using MEG slow-wave source imaging to localize affected areas and supports MEG as a tool for assisting the diagnosis of mTBI.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of sustained physical, cognitive, emotional, and behavioral deficits in the civilian population (due to motor vehicle accidents, sports, falls, and assaults) and military personnel (with blast injury as an additional cause). An estimated 5.3 million Americans live with disabilities associated with a TBI (Thurman et al., 1999). The majority of TBIs are in the "mild" range of severity. Mild TBI (mTBI) accounts for 75% of civilian TBIs (Centers for Disease Control, Prevention, National Center for Injury Prevention, Control, 2003), and 89% of active-duty military personnel and Veterans wounded in combat in Iraq and Afghanistan with combat-related TBIs

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(MacGregor et al., 2011). However, the pathophysiology of mTBI is not completely understood and the long-term effects of mTBI are controversial. Post-concussive symptoms (PCSs) in mTBI often resolve within three months after injury in the majority of individuals (Levin et al., 1987; Rutherford, 1989). However about 20% (varying from 8 to 33%) of mTBI patients show persistent long-term cognitive and/or behavioral impairments (Alexander, 1995; Binder, 1986; Binder, 1997; Bohnen et al., 1992; Rimel et al., 1981; Rutherford, 1989). At present, it is unclear why similar acute mTBI events can lead to dramatic neurobehavioral decompensation with persistent PCS in some individuals, but not in others (Jeter et al., 2013). It is also unclear what the optimal rehabilitation treatments are for mTBIs, partially due to the limited or lack of information about the loci of the injury.

Conventional neuroimaging techniques have limited sensitivity to detect physiological alterations caused by mTBI and are usually not used to assess the efficacy of mTBI treatments. Mild (and some moderate) TBI can be difficult to detect because the injuries are often not visible on conventional acute MRI or CT (Bigler and Orrison, 2004; Johnston et al., 2001; Kirkwood et al., 2006). Approximately 80% of all civilian patients with TBI do not show visible lesions using conventional MRI or CT (Alexander, 1995). Intracranial lesions in mTBI were detected by conventional neuroimaging techniques in only 4%, 16%, and 28% of civilian patients with Glasgow Coma Scale (GCS) scores (Teasdale and Jennett, 1974) of 15, 14, and 13, respectively (Culotta et al., 1996). The diagnosis of combat-related mTBI is also based primarily on the characteristics of the acute clinical sequelae following the injury; and subtle, scattered and varied lesion(s) that usually go undetected by conventional CT (Van Boven et al., 2009). The absence of abnormalities on conventional neuroimaging techniques in the majority of mTBI patients, even with persistent PCS and cognitive and/or behavioral deficits highlights the limited diagnostic and prognostic value of conventional CT and MRI.

Usually, diffuse axonal injury (DAI) is a major contributor to the PCS and cognitive deficits in mTBI patients. DAI is commonly induced by sudden acceleration–deceleration or by rotational forces. In a rodent TBI model, a silver staining technique revealed that axonal injury was the most prominent feature following blast exposure (Garman et al., 2011). In humans, the subsequent tissue injury is characterized by axonal stretching, inflammation, disruption, and separation of nerve fibers in white matter (WM), although complete axotomy has been found to be relatively rare in even severe TBI (Adams et al., 1989; Basser and Pierpaoli, 1996; Gennarelli et al., 1982; Niogi et al., 2008a; Niogi et al., 2008b; Xu et al., 2007). Conventional CT and MRI are primarily sensitive to blood from nearby torn capillaries, and less sensitive to axonal damage itself, hence they underestimate the presence of DAI, especially in mTBI cases.

Magnetoencephalography (MEG) is a non-invasive functional imaging technique that directly measures the neuronal current in gray matter (GM) with high temporal resolution (<1 ms) and spatial localization accuracy (2–3 mm at cortical level) (Leahy et al., 1998). MEG studies from Lewine et al., and our laboratory showed that MEG is highly sensitive to abnormal slow-wave signals (delta-band 1–4 Hz, and extends to theta-band 5–7 Hz) resulting from axonal injuries (Huang et al., 2009; Huang et al., 2012; Lewine et al., 1999; Lewine et al., 2007). Neurophysiological studies in animals have established a solid connection between pathological delta-wave generation in GM and axonal injuries in WM (Ball et al., 1977; Gloor et al., 1977), showing that cortical deafferentation caused by axonal injury in WM is an important factor in delta-wave production in GW. We have reported that abnormal MEG slow-waves in mTBI are related to diffusion tensor injury (DTI) abnormalities in underlying WM tracts (Huang et al., 2009). Using a region of interest (ROI) automated approach, we also detected abnormal slow-waves in 87% of patients with persistent PCS in chronic and sub-acute phases of mTBI (Huang et al., 2012). The main limitations of the ROI-based MEG approach were: 1) the limited spatial resolution defined by the size of the ROIs, and 2) the volume of the ROI varied considerably which caused variable sensitivity in detecting abnormal slow-waves in mTBI.

Voxel-based source imaging approach has the potential of overcoming the limitation of the ROI-based approach. In a study by Wienbruch (2007), a voxel-based dipole location density function approach with Z-score statistics was used for assessing resting-state MEG brain rhythms in human. Building upon previous work in this area, the present study introduces a new automated voxel-based whole-brain MEG slow-wave imaging approach for detecting abnormality on a single-subject basis for individuals with mTBI. The voxel-based MEG source images are obtained using our recent Fast-VESTAL method (i.e., Fast VEctor-based Spatio-Temporal Analysis of L1-minimum) (Huang et al., 2014) for analyzing resting-state MEG data. The goals for the present study are to: 1) establish and evaluate a normative database for the voxel-based whole-brain MEG slow-wave imaging approach; 2) examine the positive detection rates of this new approach for its ability to detect abnormality in patients with mTBI on a single-subject-basis; and 3) study the spatial distribution of abnormal MEG slow-wave loci in both individual patients and on a group basis to identify the brain areas that are particularly vulnerable to mTBI.

2. Methods and materials

2.1. Research subjects

Eighty-four (84) mTBI patients who had a chronic/sub-acute TBI (4 weeks to 5 years, mean 8.7 ± 7.3 months post-injury) with persistent ongoing PCS participated in this study. The mTBI patients were divided into two groups: the mild blast-induced TBI group consisted of 36 mTBI patients (active-duty military service members and OEF/OIF Veterans) with injuries caused by blast exposure during combat (age 28.3 ± 5.4 years, all males) while the non-blast mTBI group comprised 48 mTBI civilian patients injured due to non-blast causes (i.e., motor vehicle accidents, sports, and falls; age 30.2 ± 10.2 years, 34 males). One essential step in identifying individual TBI patients with abnormal MEG slow-waves is to first create an age-matched normative database (see below). For that purpose, 79 healthy control subjects (68 civilians and 11 active-duty military service members) with no significant history of concussion were recruited into the study (age 28.4 ± 8.7 years, 67 males). There were no statistically significant age differences between the healthy control group and either of the TBI groups. All participants gave written informed consent for study procedures, which were reviewed and approved by institutional review boards of the VA San Diego Healthcare System and Naval Health Research Center at San Diego. The informed consent followed the ethical guidelines of the Declarations of Helsinki (sixth revision, 2008) and additional research requirements for active-duty military personnel and veterans.

All mTBI patients were evaluated in a clinical interview to document the nature of the injuries and on-going PCS. The diagnosis and classification of mTBI patients were based on standard VA/DOD diagnostic criteria. Inclusion in the mTBI patient group required a TBI that met the following criteria: 1) a loss of consciousness (LOC) < 30 min or transient confusion, disorientation, or impaired consciousness immediately after the trauma; 2) post-traumatic amnesia (PTA) < 24 h; 3) an initial Glasgow Coma Scale (GCS) (Teasdale and Jennett, 1974) between 13 and 15 (if available). Since the GCS assessment was often not available in theater, military personnel (and some civilians) with missing GCS, but who met other inclusion criteria, were also recruited.

We examined PCS in all mTBI patients (based on a clinical interview). The symptoms were coded as "1" for the existence of symptoms and "0" for the absence of symptoms in 21 categories, modified slightly from the Head Injury Symptom Checklist (HISC, (McLean et al., 1984): 1) headaches, 2) dizziness, 3) fatigue, 4) memory difficulty, 5) irritability, lack of patience, lose temper easily, 6) anxiety, 7) trouble with sleep, 8) hearing difficulties, 9) blurred vision or other visual difficulties, 10) personality changes (e.g., social problems), 11) apathy, 12) lack of

spontaneity, 13) affective lability (quickly-changing emotions), 14 depression, 15) trouble concentrating, 16) bothered by noise, 17) bothered by light, 18) coordination and balance problems, 19) motor difficulty, 20) difficulty with speech, 21) numbness/tingling.

Tertiary injuries were common in patients with blast-related mTBI. The tertiary injuries involved a fall, hitting other objects (e.g., hitting parts of vehicle when the driving vehicle was hit by an IED), or being hit by other flying objects following the initial blast (Cernak and Noble-Haeusslein, 2010; Elder et al., 2010). Among our 36 blast mTBI patients, 25 also reported having tertiary injuries; 5 reported no-tertiary injuries; 6 were unsure. We use the term "blast-induced mTBI" or simple "blast mTBI" throughout this study to represent the group with combined primary blast and tertiary injuries. In the mTBI group, 2 patients had positive findings on conventional MRI (nonspecific mild white-matter T2-prolongation, not definitely related to trauma) and none had evidence of intracranial hemorrhage/hemosiderin during the chronic phase (i.e., >6 months post-injury). No healthy control subjects showed positive findings on conventional MRI. Among all mTBI patients, 27 had multiple TBIs (14 from the blast group and 13 from the non-blast group). It is not our intention in this study to use MEG to distinguish new from old neuronal injuries due to multiple TBIs. Patients with multiple TBIs were included in the analysis, and a history of the most recent and all prior TBIs was documented for further exploration. It is possible that in patients with multiple TBIs, both the old and new injuries contributed to deafferentation, thus generating abnormal MEG slow-waves.

Exclusion criteria for study participation were as follows: 1) other neurological, developmental or psychiatric disorders (e.g., brain tumor, stroke, epilepsy, Alzheimer disease, or schizophrenia, bipolar disorder, or history of learning disability). Additionally, participants with a diagnosis of post-traumatic stress disorder (PTSD) or major depression disorder (MDD) were excluded based on DSV-5 criteria and for PTSD, a Clinician Administered PTSD scale score ≥30; 2) substance or alcohol abuse according to DSM-V criteria within the six months prior to the study; 3) history of metabolic or other diseases known to affect the central nervous system (see Dikmen et al., 1995 for similar criteria); 4) extensive metal dental hardware (e.g., braces and large metal dentures; fillings are OK) or other metal objects in the head, neck, or face areas that cause non-removable artifacts in the MEG data; 5) participants taking certain medications (e.g., some sedative neuroleptics and hypnotics) known to increase delta-wave power (Niedermeyer and Lopes da Silva, 2005) were excluded from participation; 6) potential subjects were administered the Beck Depression Inventory (BDI-II) to evaluate level of depressive symptoms, and suicidal ideation; any participant who reports a "2" or "3" on the BDI-II: item 9 (suicidal thoughts or wishes) were also excluded. However, depression symptoms following mTBI are common (Rapoport, 2012); therefore, in this study, we included subjects with depression symptoms reported after their injury, but not serious enough to be diagnosed with MDD.

2.2. MEG data acquisition and signal pre-processing to remove artifacts

Resting-state MEG data (spontaneous recording for detecting MEG slow-wave signals) were collected using the VectorView™ whole-head MEG system (Elekta-Neuromag, Helsinki, Finland) with 306 MEG channels in upright position inside a multi-layer magnetically-shielded room (IMEDCO-AG) (Cohen et al., 2002) at the UCSD MEG Center. The recording was divided into three 5-minute blocks with eyes closed, alternating with three 5-minute blocks with eyes open. In the eyes-closed condition, the subject was instructed to keep the eyes closed and empty his/her mind. In the eyes-open condition, the subject was instructed to fix the eyes on a fixation point and empty his/her mind. The order of blocks was counter-balanced between subjects. Data were sampled at 1000 Hz and were run through a high-pass filter with a 0.1 Hz cut-off, and a low-pass filter with a 330 Hz cut-off. Eye blinks, eye movements, and heart signals were monitored. Precautions were taken to ensure head stability; foam wedges were inserted between the subject's head and the inside of the unit, and a Velcro strap was placed under the subject's chin and anchored in superior and posterior axes. Head movement across different sessions was about 2–3 mm. Since the MEG eyes-open data were contaminated with eye-blinks in many subjects, we focused on analyzing the eyes-closed data in the present study.

To help ensure that subjects were alert during the MEG recordings, prior to all of the study sessions, participants completed a questionnaire about the number of hours they slept the previous night, how rested they felt, and if there was any reason that they might not be attentive and perform to the best of their abilities (due to headache, pain, etc.). Participants were scheduled early in the day to avoid fatigue from performing daily activities. In addition, eyes closed sessions were rotated with eyes open sessions to monitor the amount of eye blinking and eye movement, which MEG technicians monitor online to gage the cognitive state of subjects. MEG technicians also monitored online the amount of alpha band oscillations, which is consistently associated with tonic alertness. Participants were viewed on a camera, which also allowed for MEG technicians to monitor alertness of each subject.

MEG eyes-closed data were first run through MaxFilter, also known as signal space separation, (Song et al., 2008; Taulu et al., 2004a; Taulu et al., 2004b) to remove external interferences (magnetic artifacts due to metal objects, strong cardiac signals, environment noises, etc.), and to co-register the MEG data by removing the small head movements across the three 5-min eyes-closed sessions. Next, residual artifacts near the sensor array due to eye movements and residual cardiac signals were removed using Independent Component Analysis. The software is our customized version of ICALAB (bsp.brain.riken.jp/ICALAB/).

2.3. Structural MRI, MEG-MRI registration, BEM forward calculation

Structural MRI of the subject's head was collected using a General Electric 1.5T Excite MRI scanner. The acquisition contains a standard high-resolution anatomical volume with a resolution of $0.94\times0.94\times1.2$ mm^3 using a T1-weighted 3D-IR-FSPGR pulse sequence. To co-register the MEG with MRI coordinate systems, three anatomical landmarks (i.e., left and right pre-auricular points, and nasion) were measured for each subject using the Probe Position Identification system (Polhemus, USA). By identifying the same three points on the subject's MR images using MRILAB (Elekta/Neuromag), a transformation matrix involving both rotation and translation between the MEG and MR coordinate systems was generated. To increase the reliability of the MEG-MR co-registration, approximately 80 points on the scalp were digitized with the Polhemus system, in addition to the three landmarks, and those points were co-registered onto the scalp surface of the MR images. The T1-weighted images were also used to extract the brain volume and innermost skull surface (SEGLAB software developed by Elekta/Neuromag). Realistic Boundary Element Method (BEM) head model was used for MEG forward calculation (Huang et al., 2007; Mosher et al., 1999). The BEM mesh was constructed by tessellating the inner skull surface from the T1-weighted MRI into ~6000 triangular elements with ~5 mm size. A cubic source grid with 5 mm size was used for calculating the MEG gain (i.e., lead-field) matrix, which leads to a grid with ~10,000 nodes covering the whole brain. Other conventional MRI sequences typical for identifying structural lesions in TBI patients were also performed: 1) Axial T2*-weighted; 2) axial fast spin-echo T2-weighted; and 3) axial FLAIR; These conventional MRIs were carefully reviewed by a Board-certified neuroradiologist (R.R. Lee) to determine if the subject had visible lesions on MRI.

2.4. MEG slow-wave source magnitude imaging using Fast-VESTAL

The voxel-based MEG source magnitude images were obtained using our recent high-resolution Fast-VESTAL MEG source imaging

method (Huang et al., 2014). The Fast-VESTAL technique consists of two steps. First, L1-minimum-norm MEG source images were obtained for the dominant spatial (i.e., eigen-) modes of sensor-waveform covariance matrix. Next, accurate source time-courses were obtained using an inverse operator constructed from the spatial source images of Step 1. This approach has been successfully used to obtain comprehensive MEG source-magnitude images covering the entire brain for different frequency bands of resting-state brain rhythms (Huang et al., 2014).

In the present study, each of the artifact-free, 5-minute long, eyes-closed, resting-state MEG sensor-space data were run through a band-pass filter with the passing band at 1–4 Hz (delta-frequency band). After concatenating the three sets of 5-minute band-passed filtered MEG signal, the sensor-waveform covariance matrix was calculated. Using such a covariance matrix, MEG slow-wave source magnitude images that cover the whole brain were obtained for each subject following the Fast-VESTAL procedure (Huang et al., 2014). An Objective Pre-whitening Method was applied to remove correlated environmental noise and objectively select the dominant eigen-modes of sensor-waveform covariance matrix (Huang et al., 2014).

2.5. Establishing voxel-based normative database for MEG slow-wave magnitude imaging

The MEG data processing stream in healthy control subjects includes the following steps: 1) MEG source magnitude imaging volumes obtained from Fast-VESTAL that cover the whole brain for the 1–4 Hz signals from each of the 79 healthy control subjects were first spatially smoothed using a Gaussian kernel with pre-defined full width half maximum (FWHM), and then co-registered to an MNI-152 brain-atlas template with 2 mm voxel size using FLIRT program in FSL software package (www.fmrib.ox.ac.uk/fsl/). 2) For each voxel in the MNI space, the MEG source magnitude data were first run through a logarithm transformation and then fit with a linear regression model for age and gender. The linear fitting parameters for age and gender were saved for each voxel, as parts of the normative database. 3) After adjusting for the age and gender variables, mean values and standard deviations (SD) were calculated for each voxel to form the key features of the normative database. Kolmogorov–Smirnov (K–S) tests were performed for each voxel to test for Gaussian distribution in the normative database. A "normative mask" containing all voxels that survived the K–S Gaussian distribution tests was created for the normative database. Voxels outside such a mask were not included for further analysis. 4) The source magnitude images were then converted into Z-score images using the mean values and SDs from the normative database. 5) A standard cluster analysis was performed for each Z-score imaging volume to control for family-wise errors, using "3dFWHMx" and "3dClustSim" functions in AFNI (http://afni.nimh.nih.gov). A voxel in subject's brain was considered to have statistically abnormal slow-waves if it was part of a Z-score cluster ($Z > 2$ for all voxels in the cluster) with the size equal or greater than the thresholding cluster-size (R_c) provided by "3dClustSim". The cluster-size associated with a corrected $p = 0.01$ threshold was used in the analysis. 6) For each voxel, a cluster-wise Z-score (Z_c) which was the mean value of Z-score across all neighboring voxels within R_c was calculated. The maximum value of the cluster-wise Z-score (Z_{cmax}) across the whole brain volume was obtained for each subject. Investigations were conducted to determine the optimal smoothing factor in the pre-defined FWHM, which affected R_c and Z_{cmax}.

2.6. Detecting single-subject-based abnormal MEG slow-waves in mTBI patients

We developed an approach to identify areas that generate abnormal MEG slow-wave on a single-subject basis. For each mTBI patient (blast or non-blast), the MEG source-magnitude-imaging volume was processed following Steps 1 and 2 in previous section. Then the result was run through the normative mask and then processed to adjust for the age and gender using the previously saved linear fitting parameters from normative database. Next, the resulting imaging volume was converted into a Z-score imaging volume using the mean values and SDs from the healthy control database (Step 4 in previous section). Clusters of voxels with abnormal slow-wave generations were identified using Steps 5 and 6 in previous section, and Z_{cmax} across the whole brain volume was obtained for each subject. Since the brain areas injured by TBI are highly heterogeneous with high variability across individuals, and often without global effect. Using the Z_{cmax} value (across the whole brain) is equivalent to examining the hypothesis that at least one area shows abnormal slow-waves.

We assessed the sensitivity and specificity of MEG using the Z_{cmax} measure and estimated its optimal cutoff. The standard Youden's index (i.e., sensitivity + specificity − 1) (YOUDEN, 1950) was used to calculate the optimal cutoff point (threshold of Z_{cmax}) for diagnosing mTBI using MEG slow-wave measure. The optimal cutoff is usually around the peak of a curve in which the Youden's index was plotted against different cutoff values.

2.7. Assessing the spatial distribution of abnormal MEG slow-wave generation to identify the brain areas that are vulnerable to mTBI

In addition to the single-subject-based analysis, we also performed an analysis to identify common brain areas that were likely to generate abnormal MEG slow-waves in mTBI. In this approach, MEG source imaging volume in MNI space from each mTBI patient was converted to a binary imaging volume: value "1" was assigned to the voxels showing statistically significance based on cluster-analysis in a single-subject-based analysis, and "0" to the rest of the voxels. The binary imaging volumes from all mTBI patients were summed up in the MNI space, and then the result was divided by the total number of mTBI patients to create a spatial map for the likelihood of the abnormal MEG slow-wave generation.

2.8. Assessing the effect of different spatial smoothing factors

The spatial smoothing with a Gaussian smoothing kernel may also play an important role to the positive detection rates of abnormal MEG slow-wave source imaging. Due to the nature of high heterogeneity for the location of the abnormal slow-wave generators in mTBI, overly smoothing the MEG Fast-VESTAL result is expected to decrease the sensitivity (i.e., positive detection rate) of the method. On the other hand, under-smoothing or no-smoothing may cause many voxels of the brain in the healthy control database to fail the K–S test for Gaussian distribution, thus miss some key areas of abnormal slow-wave generation in mTBI patients. The best smoothing factor is the one that can balance the above two factors, i.e., having the majority of the voxels in the healthy control database that pass the K–S test for Gaussian distribution, while maintaining high positive detection rates for abnormal MEG slow-waves in patients with mTBI.

2.9. Correlational analyses of MEG slow-wave measures and PCS

Correlation analyses were performed to examine the neuronal correlates of MEG slow-wave generation and PCS scores in patients with mTBI. The MEG slow-wave measures include the Z_{cmax} value and voxel-based MEG source magnitude Z values in MNI-152 atlas coordinates, after correction for age and gender. The PCS scores were the HISC symptom categories. The voxel-based analysis may provide important spatial information of the slow-wave generation related to each PCS category. False discovery rate (FDR) controlled family-wise error (Benjamini and Hochberg, 1995) with corrected $p < .05$. To examine potential differences between blast versus non-blast causes, correlational analyses were performed separately for the blast mTBI and non-blast mTBI groups.

3. Results

3.1. Positive detection rates of MEG slow-wave imaging for different groups of mTBI patients

MEG source magnitude images obtained from Fast-VESTAL in the 79 healthy control subjects were used to establish the voxel-based whole-brain normative database in MNI space. We examined the effects of different spatially smoothing factors by applying Gaussian smoothing kernels with different FWHMs at 2 mm, 3 mm, and 8 mm respectively. Logarithm transformation was performed for the MEG source magnitude images, and the effects of age and gender were regressed out when constructing the normative database (see Methods and materials section). Fig. 1 showed all brain voxels in the normative databases with different smoothing factors that survived the K–S test for Gaussian distribution with the alpha value of 0.05. For a smoothing kernel of 2 mm FWHM, many cortical voxels did not meet the requirement of K–S test for Gaussian distribution, indicating under-smoothing. In contrast, for a smoothing kernel of 3 mm FWHM, the majority of brain areas in the normative database met the requirements of the K–S test. Some deep brain areas did not satisfy the requirement of Gaussian distribution for this smoothing kernel. This smoothing kernel provided the best positive detection rates of abnormal MEG slow-waves in mTBI (see below). For a smoothing kernel of 8 mm FWHM, almost the entire brain met the requirement of K–S test for Gaussian distribution. However, the detection rates of MEG abnormal slow-waves decreased using such a kernel (see result below), which indicated over-smoothing.

Fig. 2 shows the Z_{cmax} values (see Methods and materials section) obtained from MEG source magnitude source imaging, plotted separately for 1) healthy control, 2) mild blast-induced TBI, and 3) mild non-blast TBI. There was minimal overlap of the Z_{cmax} values between each TBI group and the healthy control group, with the patients in all TBI groups showing markedly higher slow-wave Z_{cmax} values than the healthy control subjects. Such results provide the foundation for assessing abnormality in mTBI using MEG slow-wave source imaging on a single-subject basis.

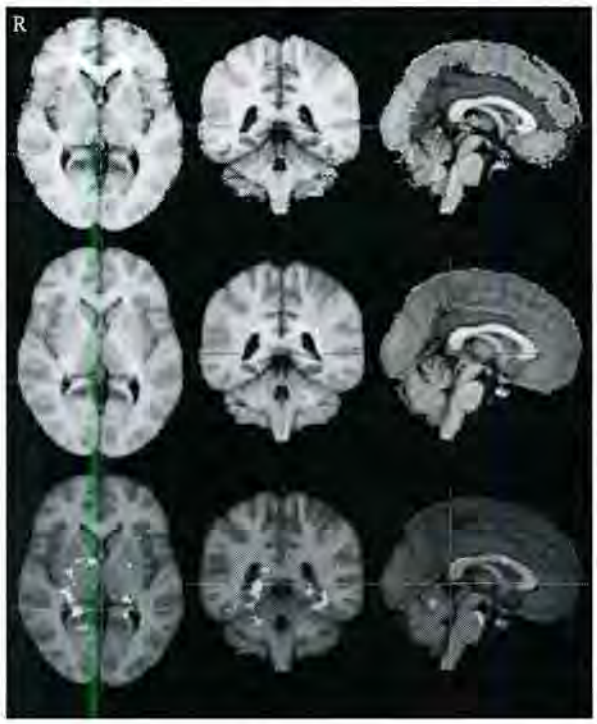

Fig. 1. Brain voxels that survived the K–S test for Gaussian distribution in the normative MEG slow-wave database. Top row (yellow) was for 2 mm FWHM, middle row (green) for 3 mm FWHM, and bottom row (blue) for 8 mm FWHM. Left column (transverse plane), middle column (coronal plane), right column (sagittal plane).

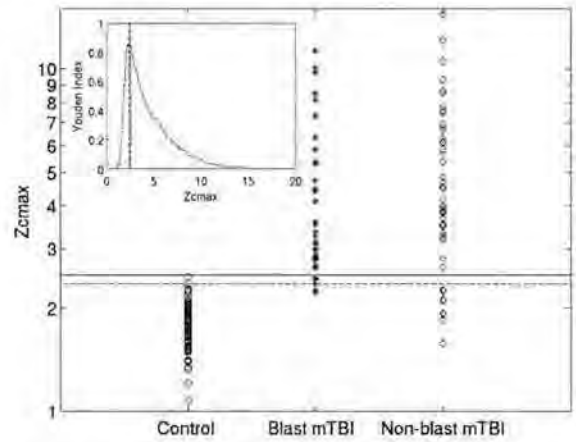

Fig. 2. Z_{cmax} values obtained from MEG source imaging for 1–4 Hz are plotted separately for 1) healthy control, 2) mild blast-induced TBI, and 3) mild non-blast-induced TBI, groups respectively. The embedded plot: the Youden index is plotted as a function of the Z_{cmax} cutoff. The solid and dashed lines in both plots indicate cutoff values of 2.50 and 2.35, respectively.

The optimal cutoff (threshold) for Z_{cmax} was obtained from the Youden's index curve (embedded plot in Fig. 2) using 79 healthy controls and 84 mTBI patients (blast plus non-blast). The cutoff value associated with the peak of the Youden's index was 2.35 (dashed lines in Fig. 2 and embed) which corresponded to specificity (1 − false-positive rate) of 98.7%. We chose a little more conservative cutoff value of 2.50 (solid lines in Fig. 2 and embed) which corresponded to specificity of 100% (i.e., 0 false positive rate, no healthy control subjects showed Z_{cmax} value above this threshold). With this threshold (solid horizontal line in Fig. 2), the positive detection rates (i.e., sensitivity values) were 86.1%, 83.3%, and 84.5% for blast-induced, non-blast, and combined (blast-induced plus non-blast) mTBI groups, respectively.

With such positive detection rates of the MEG slow-wave source imaging approach, the difference between each mTBI group and the healthy control group was expected to be highly significant (but not necessarily among different mTBI groups). Two-tailed t-tests confirmed that in comparison to the healthy control group, the Z_{cmax} values are indeed significantly higher in the mild blast-induced TBI ($t = 9.3$, $p < 10^{-14}$), and in the mild non-blast TBI ($t = 10.4$, $p < 10^{-17}$) groups. However, there were no significant differences in the Z_{cmax} values between the two mTBI groups.

3.2. Results from individual mTBI cases using single-subject-based analysis

Although the analysis using Z_{cmax} provides crucial information for positive detection rate that may assist in diagnosis, it does not address the loci and characteristics of abnormal slow-wave generation in individual TBI patients. The voxel-based framework based on Fast-VESTAL MEG source images (see Methods and materials section) provides a viable single-subject-based analysis for identifying the sources of abnormal MEG slow-wave generation in individual mTBI patients. Fig. 3 shows the results of single-subject-based analysis revealing statistically abnormal MEG slow-wave generation from 6 representative mTBI cases. The results were shown in MNI space. The abnormal MEG slow-wave sources were heterogeneous in locations across these mTBI patients. In Case 1, single-subject-based analysis showed abnormal MEG slow-waves from two right superior frontal areas. In Case 2, the abnormal slow-waves were from right dorsal–lateral pre-frontal cortex (DLPFC) and right ventral temporal pole areas. In Case 3, bilateral frontal pole, DLPFC, and right occipital areas showed abnormal slow-waves. In Case 4, two areas within left DLPFC and one area in ventral posterior temporal lobe generated abnormal MEG slow-waves. In Case 5,

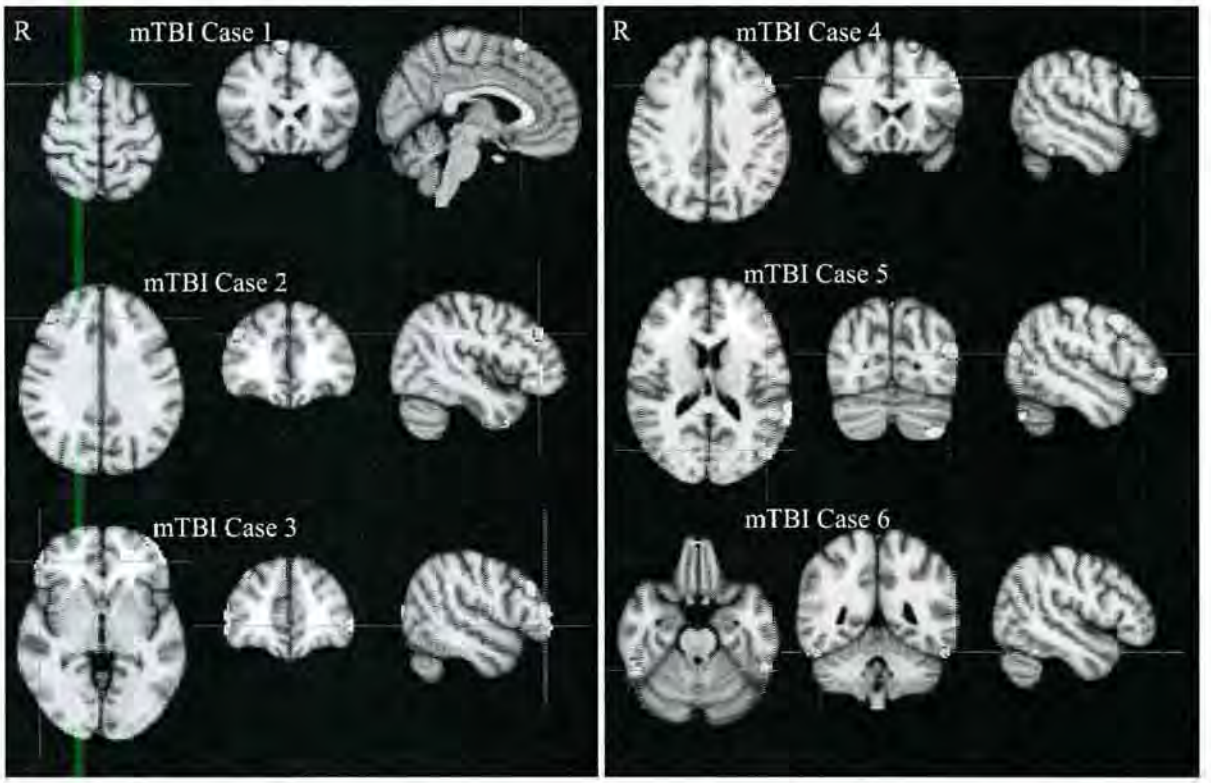

Fig. 3. Single-subject-based analysis showing statistically abnormal MEG source-wave sources in representative mTBI cases. Left column (transverse plane), middle column (coronal plane), right column (sagittal plane).

posterior parietal lobe, DLPFC, frontal pole (FP), and cerebellum, all in right hemisphere generated abnormal slow-waves. Finally, bilateral inferior temporal lobe and midline orbital frontal cortex (OFC) showed abnormal slow-waves in Case 6.

3.3. Percent likelihood maps of abnormal MEG slow-wave generation in mTBI

Although the location of slow-wave generation is highly heterogeneous in locations across mTBI patients, analysis was performed to identify common brain areas that likely generate abnormal MEG slow-waves in mTBI, by following the procedure described previously in Methods and materials section. The percent likelihood maps of abnormal MEG slow-wave generation shown in Fig. 4 revealed that the overall percent likelihood level from any specific brain area was low (5%–15%, see color scale). However, the following areas showed higher likelihood than the rest of the brain for generating abnormal slow-waves: bilateral DLPFC, bilateral ventral lateral prefrontal cortex (VLPFC), bilateral FP, right OFC, left inferior–lateral–posterior parietal lobe, bilateral inferior temporal lobes, right hippocampus, and bilateral cerebella.

3.4. The effects of over-smoothing

In the previous section we observed that the under-smoothing with a Gaussian kernel of 2 mm FWMH resulted in many voxels not surviving the K–S test for Gaussian distribution in the normative database. Here, we examined the impact of the over-smoothing to the positive detection rates in MEG slow-wave source imaging approach, using a smoothing kernel of 8 mm FWMH. With this smoothing kernel and cut-off value chosen at 100% specificity, the positive detection rates of MEG slow-wave imaging as measured by Z_{cmax} decreased to 27.7% for the blast mTBI group, 31.3% for the non-blast mTBI group, and 29.8% for the combined mTBI group. These values are markedly lower than those obtained using the 3 mm FWMH Gaussian smoothing kernel reported in previous section. Nevertheless, even with this 8 mm FWMH smoothing kernel, both the blast mTBI and non-blast mTBI groups still showed significantly higher Z_{cmax} than the healthy control group: $t = 3.8$, $p < 10^{-3}$ for blast mTBI patients versus control subjects; $t = 5.1$, $p < 10^{-5}$ for non-blast mTBI patients versus control subjects. There was no significant group difference in Z_{cmax} between blast and non-blast mTBI groups with the 8 mm smoothing kernel.

3.5. MEG slow-wave measures correlated with PCS in mTBI

Correlational analyses of MEG slow-wave measures and PCS were performed in the blast as well as non-blast mTBI groups. In the blast mTBI group, the Z_{cmax} values positively correlated with anxiety ($r = 0.41$, $p < 0.05$ uncorrected), and apathy ($r = 0.37$, $p < 0.05$ uncorrected). In the non-blast mTBI group, the Z_{cmax} values positively correlated with trouble with sleep ($r = 0.29$, $p < 0.05$, uncorrected). However, none of the correlations survived FDR correction.

In contrast, significant correlations (Fig. 5) were found with the voxel-based correlational analysis between MEG source magnitude (Z values in MNI-152 coordinates) and PCS scores. In the blast mTBI group, personality change symptoms (e.g., social problems) positively correlated with MEG slow-wave generation in bilateral OFC and ventromedial prefrontal cortex (vmPFC); trouble concentrating and affective lability (quickly-changing emotions) symptoms both positively correlated with slow-wave generation in right OFC; blurred vision or other visual difficulties symptoms positively correlated with slow-wave generation in right fusiform gurus. Fig. 5 also shows that in the non-blast mTBI group, depression symptoms positively correlated with slow-wave generation in anterior cingulate cortex (ACC). When combining

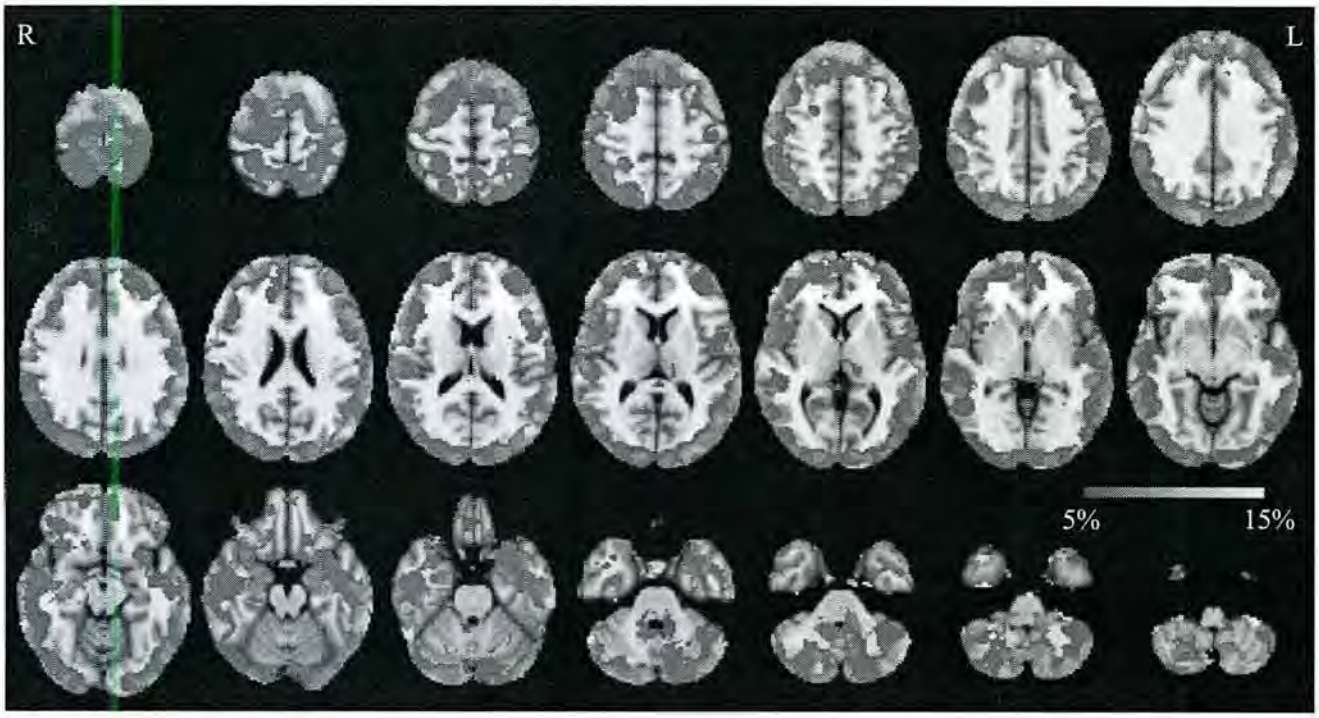

Fig. 4. Voxel-based maps showing the percent likelihood of abnormal MEG slow-wave generation across the whole brain.

the blast and non-blast mTBI groups, only MEG source magnitude from the right OFC was positively correlated with the symptoms of trouble concentrating in the combined pool (not shown). The threshold of the voxel-based analyses was at the corrected $p = 0.05$ by FDR.

4. Discussion

4.1. Detection sensitivity on an individual level

Using the automated voxel-based MEG source imaging approach (Fig. 2), we found abnormal delta-waves in 86.1% of blast mTBI, 83.3% the non-blast mTBI, and 84.5% for all mild TBI patients (blast-induced plus non-blast causes). All mTBI patients were symptomatic with ongoing PCS at the time of the MEG exam. These positive detection rates were markedly higher than the <10% rate using the conventional neuroimaging approach (i.e., MRI) in the same mTBI patients. Furthermore, the positive MRI findings in our mTBI patients could not be attributed to the head trauma alone because similar MRI abnormalities were also shown in subjects without a history of TBI. Our results are consistent with findings from previous MEG studies in mTBI using dipole fit to hand-selected slow-wave epochs (Lewine et al., 1999; Lewine et al., 2007). The resting-state MEG recording procedure is spontaneous, requires minimal effort from TBI patients, and is thus insensitive to patients' performance and effort. We controlled for any other factors that may increase slow-wave power such as neuroleptic, sedative, or hypnotic medications, sleep deprivation, as well as other neurological disorders (stroke, epilepsy, brain tumor, etc.). These results corroborate well-documented EEG findings reporting that focal delta-waves signify the presence of brain injury in alert, awake adults (Fisch, 1999; Rowan and Tolunsky, 2003). Thus, our findings underscore the diagnostic utility of our automated and voxel-based MEG slow-wave source imaging, based on Fast-VESTAL, particularly for mTBI.

4.2. MEG slow-wave activity associated with PCS

It is also interesting that the voxel-based correlational analyses (Fig. 5) showed that slow-wave generation in areas that are part of the ventral prefrontal cortex (i.e., OFC and vmPFC) positively correlated with personality change, trouble concentrating, and affective lability symptoms in the blast mTBI group. In addition, slow-wave generation from the ACC positively correlated with depression in the non-blast mTBI group. Many of these symptoms are psychiatric-based risk factors. Present findings are consistent with studies showing that mTBI increases the likelihood of developing psychiatric-based symptoms, or

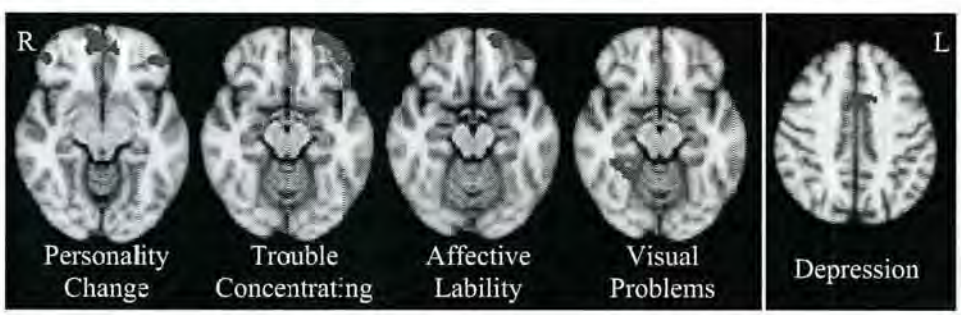

Fig. 5. MEG slow-wave source magnitude significantly correlated with PCS in blast mTBI group (first 4 panels) and non-blast mTBI group (last panel). FDR corrected $p < 0.05$.

in some patients, is associated with the development of psychiatric disorders (for reviews, see Bryant et al., 2010; Schwarzbold et al., 2008).

Present findings are also consistent with knowledge that damage to the prefrontal areas may affect executive functions, emotion, mood, as well social behavior regulation (Carlson, 2013; Kandel et al., 2000). This may be because these areas have rich connections to many cortical and subcortical areas. For example, the vmPFC is connected to and receives input from the ventral tegmental area, amygdala, temporal lobe, olfactory system, and dorsomedial thalamus. In turn, the vmPFC sends signals to amygdala, temporal lobe, lateral hypothalamus, hippocampal formation, cingulate cortex, and other regions of the prefrontal cortex (Carlson, 2013). On the other hand, the OFC shares extensive reciprocal connections with primary and associated somatosensory, auditory, and visual cortices, as well as areas in the limbic system (e.g., hippocampus, amygdala, thalamus, hypothalamus, and cingulate gyrus), and projects to the motor areas reflecting integration for executive motor control (Carlson, 2013). The abnormal slow-wave generation from the OFC that was associated with trouble concentrating may suggest a deficit of sensory integration due to mTBI. In addition, the association between slow-wave generation from the right fusiform gyrus and the symptoms of blurred vision or other visual difficulties in the blast mTBI group is consistent with studies showing that the fusiform gyrus is important in face, object, and body recognition and processing (Downing et al., 2001; Kanwisher et al., 1997; Sergent et al., 1992; Weiner and Grill-Spector, 2010). A meta-analysis showing that facial affect recognition difficulty is common after TBI (Babbage et al., 2011) is also consistent with present findings.

Using the dipole location density method, Wienbruch (2007) examined healthy subjects and reported that male subjects had significantly higher frontal-central MEG slow-wave generation near ACC than female subjects. The present study corrected for both age and gender when calculating the Fast-VESTAL source-magnitude Z scores. As such, our finding of ACC MEG slow-wave activity positively correlation with depression in the non-blast mTBI group was controlled for gender and age. Nevertheless, there were more males than females (67 versus 12) in our healthy control group (same for the two mTBI groups) when constructing our normative database. This was because we needed to balance our blast mTBI group which contained all males by using the same normative database for assessing patients in both the blast and non-blast mTBI groups. Future study with symmetrical design (more females) in all three groups will be needed to thoroughly address if and how gender modulates these findings.

Using the same dipole location density method, Rockstroh and colleagues examined MEG slow-waves in inpatients with schizophrenia and affective disorders (Rockstroh et al., 2007). They found that inpatients with schizophrenia had more slow-wave generators with maxima in frontal and central areas, whereas inpatients with affective disorder had fewer slow-wave generators in similar frontal and central regions. In the present study, MEG slow-wave activity in ACC positively correlated with depression symptoms in the non-blast mTBI patients. Although depression is a common symptom across schizophrenia, affective disorders, and mTBI, direct comparison between findings from the study by Rockstroh and colleagues and the present study is difficult due to the following two factors: 1) these are three different brain disorders; 2) all subjects with schizophrenia and affective disorder in the study by Rockstroh and colleagues were inpatients treated by a variety of medications including the neuroleptics, whereas all of our mTBI outpatients were free of sedative, neuroleptic, and hypnotic medications (see exclusion criteria). Future studies in which the effects of medications are controlled will be needed to address the correlation between abnormal slow-wave generation and common symptomology (such as depression) across different disorders.

It is not clear what accounts for the different correlation patterns between MEG slow-wave source imaging and TBI symptomatology in the blast versus non-blast mTBI groups (Fig. 5). In particular, it is not clear why more brain areas showed a significant correlation between MEG and mTBI symptoms in the blast mTBI group than in the non-blast mTBI group. We speculate that as a common cause in the former group, blast may contribute to our findings. However, future study is needed to confirm or disprove this hypothesis.

4.3. Diffused nature and "vulnerable" regions for mTBI

The present study also revealed the diffuse nature of the neuronal injuries in TBI patients (Figs. 3 and 4). Such findings are consistent with the mechanism of diffuse axonal injury in TBI due to a combination of linear and rotational acceleration and deceleration (Adams et al., 1989; Arfanakis et al., 2002; Basser, 1995; Huisman et al., 2004; Niogi and Mukherjee, 2010; Niogi et al., 2008a; Xu et al., 2007). The results are also consistent with our previous findings that abnormal MEG slow-waves are generated from cortical gray-matter areas that connect to white-matter fibers with reduced DTI fractional anisotropy due to axonal injury in patients with mTBI (Huang et al., 2009). The diffuse nature of MEG slow-wave generation is also consistent with a DTI study in blast mTBI subjects which showed reduced FA in a diffuse, widespread, and spatially variable pattern (Davenport et al., 2012).

Although the location of slow-wave generation is highly variable across mTBI patients (see Fig. 3), in the present study analysis was performed to identify common brain areas that likely generate abnormal MEG slow-waves in mTBI (see Fig. 4). Multiple regions in the frontal lobes (i.e., DLPFC, VLPFC, FP, and OFC) were more likely than other brain regions to generate abnormal MEG slow-waves, which suggested that the frontal lobe is probably the most vulnerable lobe to head trauma. In addition, the posterior parietal lobe, inferior temporal lobes, hippocampus, and cerebella also have a relatively higher likelihood for generating abnormal MEG slow-waves than other brain areas, indicating that these regions are also particularly vulnerable to head trauma. A forthcoming study that correlates the MEG slow-wave with cognitive functions in mTBI will examine the connection of slow-wave generation and abnormal brain function (Robb et al., in preparation).

4.4. Neuronal mechanisms of abnormal slow-waves

Neurophysiological studies in animals have shown that cortical deafferentation caused by axonal lesions in WM is an important factor in pathological delta-wave production in GW (Ball et al., 1977; Gloor et al., 1977). We believe that the cortical deafferentation caused by axonal injury is the main mechanism for abnormal MEG slow-wave generation in mTBI. However, pathological delta-wave production can also be induced by deafferentation following the administration of atropine in WM in animals (Schaul et al., 1978). It is known that atropine is a competitive antagonist of acetylcholine receptors and can block and/or limit the cholinergic pathway. So the electrophysiological similarity of lesion-induced and atropine-induced slow waves raises the possibility that a defect in cholinergic pathways plays a role in pathological slow-wave generation (Schaul, 1998). It is possible that the abnormal MEG slow-waves in mTBI from the present study were partially due to blockage and/or limitation of cholinergic transmission after TBI, in addition to axonal injury in WM. In the human brain, the projections of cholinergic pathways highly overlap with the WM fiber tracts (Selden et al., 1998), which make the cholinergic pathways similarly susceptible as WM tracts to rotational forces during head trauma. Like axonal injury, blockage and/or limitation of cholinergic transmission may result in cortical deafferentation and pathological slow waves that are expected to affect human brain function in mTBI patients.

Abnormal slow waves are not the only abnormal findings in TBI. A recent MEG study in a group with mixing mild, moderate, and severe TBI patients showed reduced functional connectivity primarily in bilateral frontal and left greater than right parieto-temporo-occipital regions as well as the right thalamus (Tarapore et al., 2013). Another recent MEG study in sensor space also showed a reduced level of complexity in mild TBI patients (Luo et al., 2013). In a future study, we will examine

the relationships between MEG slow-wave generation and functional connectivity in different frequency bands in mTBI.

4.5. Voxel-based versus ROI approaches

The MEG results using the new voxel-based Fast-VESTAL approach were similar to our previous ROI-approach frequency-domain VESTAL which showed positive detection rate of 87% (Huang et al., 2012), however larger groups of mTBI patients were examined in the present study. Furthermore, the voxel-based Fast-VESTAL approach overcomes the main limitations of variable sensitivity associated with our previous ROI-based approach using frequency-domain VESTAL (Huang et al., 2012). The spatial-sensitivity of the voxel-based approach is more uniformly distributed across the brain volume whereas the sizes of 96 cortical ROIs in previous ROI-based approach varied substantially from one ROI to another. Second, as shown in Fig. 3, the voxel-based MEG source images can be informative, with good spatial resolution, in assessing the abnormal slow-waves on a single-subject-basis. In mTBI patients, it was common that multiple regions generated abnormal slow-waves. It has been shown that VESTAL and Fast-VESTAL approaches can localize neuronal sources with a variety of spatial profiles (e.g., focal, multi-focal, dipolar, and distributed) and a variety of temporal profiles (e.g., uncorrelated, partially-correlated, and 100% correlated source time-courses) (Huang et al., 2006; Huang et al., 2014). Generators of abnormal slow-waves in mTBI patients can be in one or more of the above spatial-and-temporal profiles (Huang et al., 2009), and Fast-VESTAL based MEG source imaging is ideal to handle such variability. Third, the voxel-based framework of MEG source imaging using Fast-VESTAL (Huang et al., 2014) allows us to implement many imaging-processing and statistical-analysis tools from existing software packages (FSL, AFNI, Freesurfer, etc.) that were previously designed for other functional (e.g., fMRI and PET) or structural neuroimaging techniques.

4.6. Effect of spatial-smoothing factor

In the present study we have shown that the spatial smoothing factor in MEG source imaging plays an important role in the positive detection rate of abnormal slow waves. Although group differences were preserved, high spatial smoothing using 8 mm FWHM kernel markedly reduced the positive detection rate of abnormal slow waves compared with the result using the 3 mm smoothing kernel. This finding suggests that the abnormal MEG slow-wave generation may be more of a local effect, and MEG source analysis methods with high spatial resolution may be essential in detecting abnormal slow waves in mTBI. In the present study, a MEG source imaging method with high spatial resolution (i.e., Fast-VESTAL) was used to analyze resting-state MEG data in mTBI. Previous MEG studies by Lewine and colleagues used dipole modeling (another MEG source analysis with focal source modeling) and found abnormal slow waves in 65%-86% of mTBI patients (Lewine et al., 1999; Lewine et al., 2007). Despite the robust group differences in scalp EEG, the positive detection rate of abnormal slow-waves using scalp EEG was substantially lower than that with MEG (Lewine et al., 1999). Differences in positive detection rates may be due to the smearing effect of the skull tissue, which with its poor conductivity substantially distorted the electric fields and reduced the spatial resolution of the EEG signal during scalp recording; whereas, head tissues are essentially transparent to MEG signals.

4.7. MEG source imaging with Fast-VESTAL versus other approaches

In the present study, Fast-VESTAL method plays an essential role in assessing the source magnitude differences in mTBI. It was shown that Fast-VESTAL can: 1) provide high resolution source images for multiple correlated sources; 2) faithfully recover source time-courses; 3) perform robustly in poor SNR conditions; 4) handle correlated brain noise; and 5) effectively create resting-state MEG source images that are highly consistent with known neurophysiology findings (Huang et al., 2014). We have also shown that for resting-state MEG signals, the source magnitude images obtained with beamformer technique (a popular MEG source analysis method) were not as consistent with neurophysiology findings as those from Fast-VESTAL (Huang et al., 2014). This is likely due to beamformer's intrinsic limitation which assumes that the neuronal sources are uncorrelated (Robinson and Vrba, 1999; Sekihara et al., 2001; Van Veen et al., 1997), a questionable assumption when dealing with resting-state MEG signals.

Wienbruch introduced a different voxel-based resting-state MEG source analysis approach, in which a sequential single dipole model was used to fit MEG signal for each time point (i.e., single equivalent current dipoles were fitted for each time point). The dipoles with goodness-of-fit (GoF) > 0.9 were kept. Then, voxel-based dipole location density measure was used to establish a normative database, and a Z-score statistics was used to assess abnormalities. Our Fast-VESTAL source imaging approach improves upon the seminal work in this area by Wienbruch (2007) in two ways. First, the approach by Wienbruch is less able to handle time points where multiple sources contribute simultaneously to the MEG measures. For example, in many such cases, the GoF with a single sequential dipole model would be less than the 0.9 threshold, and such that those time points would be discarded from further analysis in Wienbruch's approach. With the Fast-VESTAL approach, all time points free of artifacts are used in the analysis since Fast-VESTAL is designed to model multiple highly correlated sources simultaneously. Second, the dipole location density measure from Wienbruch's approach does not directly take into consideration of the strength differences in the sequential dipoles. For example, two dipoles with different strengths (e.g., one is twice as strong as the other) that both meet the GoF threshold would contribute equally to the dipole location density measure. In contrast, Fast-VESTAL directly assesses the source magnitude differences at all grid locations, which is also a key feature that differentiates the MEG signals from one subject to another.

In the dipole-fitting approach, the basic assumption is that the neuronal generators of MEG signals are focal and can be modeled by one or a few dipoles. The dipole location and dipole moment parameters are determined by an over-determined non-linear optimization procedure. In fact, an automated multi-dipole approach "multi-start spatio-temporal" method was developed in our lab in the past to model multiple dipoles without the requirements of the initial guess of the dipole locations (Huang et al., 1998; Huang et al., 2005). However, all dipole modeling techniques require the number of dipoles to be pre-estimated, and the non-linear optimization procedure becomes extremely high in computational cost and may be trapped into local minima when the number of dipoles increases. Usually, 8–10 dipoles are the upper limit that the dipole-fitting methods can handle (Huang et al., 2005).

In the Fast-VESTAL approach, the brain volume, or just the cortex is pre-divided into a source grid with several thousand nodes, and a dipole is assigned to each grid node. Fast-VESTAL fits the MEG sensor waveforms while minimizing the total current across all grid nodes to reduce the ambiguity of the multiple plausible solutions. Fast-VESTAL identifies the grid nodes with neuronal activity with high resolution, and suppresses the magnitude at the grid nodes without neuronal activity to essentially zero (Huang et al., 2014). The Fast-VESTAL procedure is efficient in computational cost, can handle many correlated as well as uncorrelated dipolar sources, and is not trapped in the "local minima". Robust control mechanisms were built into the Fast-VESTAL algorithm to fit the brain signal and to prevent the algorithm from fitting correlated and/or uncorrelated noise (Huang et al., 2014).

In the MEG responses that are known to contain a few focal neuronal generators (e.g., in the case of human somatosensory responses evoked by median-nerve stimuli), both Fast-VESTAL and multiple-dipole fitting approaches produced sparse solutions that are very similar in location and source time-course, and both solutions are consistent with previous neurophysiological findings (Huang et al., 2005; Huang et al., 2014). In a

sense, Fast-VESTAL is a more effective and improved way in finding a sparse solution over the multiple-dipole fit. However, systematic comparisons of Fast-VESTAL, dipole-fitting methods including the single-sequential-dipole fit (Wienbruch, 2007) and multiple-dipole fit (Huang et al., 1998; Huang et al., 2005), and physiology approaches are an interesting research topic for the future, but are currently beyond the scope of the present study.

In summary, the present study examined the sensitivity of our new automated voxel-based whole-brain MEG slow-wave imaging approach based on Fast-VESTAL for detecting abnormality in patients with mild TBI on a single-subject basis. The results show that this MEG slow-wave source imaging method achieves a positive detection rate of 84.5% for the mTBI group (blast-induced plus non-blast) with the threshold chosen at a zero false positive rate. The results showed that although abnormal MEG slow-wave generations in individual mTBI patients were highly variable in space with a diffuse characteristic, the prefrontal lobe, posterior parietal lobe, inferior temporal lobe, hippocampus, and cerebella were particularly vulnerable to head trauma. The result also showed that MEG slow-wave generation in prefrontal areas positively correlated with personality change, trouble concentrating, affective lability, and depression symptoms. In addition, we found that a high spatial smoothing factor can reduce the positive detection rate of abnormal MEG slow-waves in mTBI, which suggests that MEG source analysis methods with high spatial resolution may be essential for mTBI study. We believe the potential neuronal mechanisms of MEG slow-wave generation were the deafferentations caused by axonal injury and/or blockages/limitations of cholinergic transmission in TBI. This study provides support for using MEG slow-wave source imaging to localize affected areas and highlights the potential use of this methodology for the clinical diagnosis of mTBI.

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Voxel-wise resting-state MEG source magnitude imaging study reveals neurocircuitry abnormality in active-duty service members and veterans with PTSD

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ABSTRACT

Post-traumatic stress disorder (PTSD) is a leading cause of sustained impairment, distress, and poor quality of life in military personnel, veterans, and civilians. Indirect functional neuroimaging studies using PET or fMRI with fear-related stimuli support a PTSD neurocircuitry model that includes amygdala, hippocampus, and ventromedial prefrontal cortex (vmPFC). However, it is not clear if this model can fully account for PTSD abnormalities detected directly by electromagnetic-based source imaging techniques in resting-state. The present study examined resting-state magnetoencephalography (MEG) signals in 25 active-duty service members and veterans with PTSD and 30 healthy volunteers. In contrast to the healthy volunteers, individuals with PTSD showed: 1) hyperactivity from amygdala, hippocampus, posterolateral orbitofrontal cortex (OFC), dorsomedial prefrontal cortex (dmPFC), and insular cortex in high-frequency (i.e., beta, gamma, and high-gamma) bands; 2) hypoactivity from vmPFC, Frontal Pole (FP), and dorsolateral prefrontal cortex (dlPFC) in high-frequency bands; 3) extensive hypoactivity from dlPFC, FP, anterior temporal lobes, precuneous cortex, and sensorimotor cortex in alpha and low-frequency bands; and 4) in individuals with PTSD, MEG activity in the left amygdala and posterolateral OFC correlated positively with PTSD symptom scores, whereas MEG activity in vmPFC and precuneous correlated negatively with symptom score. The present study showed that MEG source imaging technique revealed new abnormalities in the resting-state electromagnetic signals from the PTSD neurocircuitry. Particularly, posterolateral OFC and precuneous may play important roles in the PTSD neurocircuitry model.

1. Introduction

Individuals exposed to a traumatic event may develop post-traumatic stress disorder (PTSD) with debilitating post-traumatic stress symptoms, including intrusive memories, avoidance behavior, emotional numbing, and hyperarousal (American Psychiatric Association, 2004). PTSD is a major health concern that affects approximately 7.7% of Americans (Kessler et al., 1995, 2005) and is particularly prevalent among military service members who have served in combat (Dohrenwend et al., 2006; Magruder and Yeager, 2009). The recent conflicts in Iraq and Afghanistan have been no exception, with combat veterans returning with elevated rates of PTSD (Hoge et al., 2004; Smith et al., 2008; Tanielian and Jaycox, 2008).

In light of these findings, much effort has been focused on determining symptom etiology and the associated neural mechanisms of PTSD. The development of neurocircuitry models of PTSD has relied strongly on findings from pre-clinical studies of fear conditioning. Evidence from lesion studies, pharmacological manipulations, and electrophysiology in animals and humans suggest that interactions between the amygdala, ventromedial prefrontal cortex (vmPFC), and hippocampus control different aspects of fear processing (Hartley and Phelps, 2010; Rosen and Lilienfeld, 2008). The amygdala is involved in acquisition of

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fear conditioning and extinction learning, whereas the vmPFC is thought to mediate memory storage and retrieval during extinction learning. Hippocampal connections to the amygdala and vmPFC may support processing contextual information of threat-related stimuli.

Amygdala, vmPFC, and hippocampal regions implicated in preclinical fear processing are thought to be dysfunctional in PTSD (Rauch et al., 1998, 2006). Functional neuroimaging findings using positron emission topography (PET) and functional magnetic resonance imaging (fMRI) suggest that individuals with PTSD exhibit hyperresponsive amygdala activity to trauma or fear-related stimuli (Shin and Liberzon, 2010), during emotionally neutral tasks (Bryant et al., 2005; Shin et al., 2004b), and even at rest (Chung et al., 2006; Semple et al., 2000). A hyperresponsive amygdala contributes to the exaggerated fear response characteristic of PTSD (Anderson et al., 2003). Conversely, PTSD has been associated with a hyporesponsive vmPFC (Hughes and Shin, 2011). A hyporesponsive PFC, as well as reduced connectivity to the amygdala (Jin et al., 2013; Shin et al., 2004a) may indicate insufficient inhibitory control over exaggerated fear responses. Lastly, abnormal hippocampal function (Corcoran and Maren, 2001) and reduced connectivity to the amygdala (Dolcos et al., 2004; McGaugh, 2004) may be associated impairments in contextual memory processing and the ability to inhibit intrusive memories (Shin et al., 2004a), although findings have been mixed (Hughes and Shin, 2011). A recent resting-state fMRI study showed increased activity in amygdala and reduced spontaneous neural activity in the dlPFC, but no abnormal decrease of resting-state fMRI activity in the vmPFC (Yan et al., 2013).

Neuroimaging studies using PET and fMRI have contributed greatly to understanding PTSD neurocircuitry in humans; however, these techniques measure metabolic and hemodynamic changes which reflect neuronal activity indirectly (Logothetis, 2003). In addition, PET and fMRI techniques have limited temporal resolution (minutes to seconds) and consequently limited coverage and resolution in the frequency domain. Since neurons communicate to each other via exchanging electric current signals, direct electrophysiological measures are required to study neurophysiological processes that are associated with these hemodynamic signals (Scholvinck et al., 2013). PET and fMRI studies also have implicated different neural pathways that may be hyporesponsive in PTSD; thus, there is some remaining discrepancy whether PTSD is associated with reduced activity in the vmPFC or dlPFC pathways. Furthermore, although the orbitofrontal cortex (OFC) is usually considered to be part of the extended limbic system, the contribution of OFC to PTSD has not been fully elucidated.

Electromagnetic measures such as magnetoencephalography (MEG) provide direct measurements of neuronal activity with millisecond temporal resolution. Using a single dipole model, Kolassa and colleagues reported elevated production of focally generated slow waves (1–4 Hz) in PTSD, particularly in left temporal brain regions, with peak activities in the region of the insula. Using a MEG sensor-space synchronous neural interactions analysis, Georgopoulos, Engdahl, and their colleagues correctly classified individuals with PTSD and healthy control subjects with >90% overall accuracy of classification (Georgopoulos et al., 2010). They also found differences in MEG communication measures between temporal and parietal and/or parieto-occipital right hemispheric areas with other brain areas in PTSD (Engdahl et al., 2010). However in sensor space, it is difficult to determine whether the structures identified by PET and fMRI in PTSD neurocircuitry generate abnormal electromagnetic activity. Namely, whether electromagnetic-based source imaging techniques will lead to similar or different findings from those obtained in PET and fMRI in PTSD neurocircuitry has largely been unexplored.

In the current study, we examined neural activity associated with PTSD using resting-state MEG. MEG is a non-invasive functional imaging technique that directly measures magnetic signals generated by neuronal current in gray matter with high temporal resolution (<1 ms) and spatial localization accuracy (2–3 mm at cortical level) (Leahy et al., 1998). MEG's high temporal resolution directly translates into a wide range of coverage for the neuronal magnetic signals in the frequency domain, which is usually divided into different frequency bands. MEG's insensitivity to the electric conductivity profile of the head tissue makes it a better technique than electroencephalography (EEG) in localizing neuronal sources. Our newly developed high-resolution MEG source imaging method called Fast-VESTAL allowed us to perform voxel-wise whole-brain source imaging of human brain rhythms in healthy volunteers (Huang et al., 2014a), and makes MEG source imaging a good candidate for localizing abnormal electromagnetic signals in disorders such as PTSD. The primary goal for this study was to examine if the existing PTSD neurocircuitry model including the amygdala, vmPFC, and hippocampus can account for abnormalities detected directly by electromagnetic-based source imaging techniques in resting-state. To achieve this goal, we used high-resolution MEG source imaging technique for direct examination of neuronal activity in PTSD, especially in the areas that we think to be abnormal, i.e. amygdala, vmPFC, OFC, hippocampus, dlPFC, dmPFC including dorsal anterior cingulate cortex (dACC), insular cortex, and precuneous. In addition, using MEG, we are able to explore potential MEG abnormalities in different frequency bands which are associated with different neuronal mechanisms (see Discussion), and compare MEG findings with previously published results from other functional imaging techniques that have been used to study PTSD.

2. Materials and methods

2.1. Research participants

Twenty-five participants (24 males, 1 female; mean [SD] age: 31.0 [5.5]) with PTSD took part in this study. Among these participants, 10 were active-duty Marines and Sailors from Camp Pendleton and Naval Medical Center in San Diego, and 15 were adult outpatient OEF/OIF Veterans recruited from VA San Diego Healthcare System. Mean [SD] years of education for the participants with PTSD were 13.2 [1.4]. All participants gave written informed consent for study procedures, which were reviewed and approved by institutional review boards of the VA San Diego Healthcare System and Naval Health Research Center at San Diego. The informed consent followed the ethical guidelines of the Declarations of Helsinki (1975) and additional research requirements for active-duty military personnel and veterans.

Symptoms of PTSD were assessed using the Clinician Administered PTSD Scale (CAPS) (Blake et al., 1995) or the Post-traumatic Stress Disorder Checklist (PCL) (Weathers et al., 1999) in accordance with the criteria from the Diagnostic and Statistical Manual of Mental Disorders IV-TR (American Psychiatric Association, 2000). A total of 18 participants met the criteria for PTSD and 7 met the criteria for partial PTSD. Participants who completed the CAPS met the criteria for PTSD ($n = 14$) if they reported at least 1 re-experiencing symptom, 3 avoidance symptoms, and 2 hyperarousal symptoms; patients met the criteria for partial PTSD ($n = 5$) if they reported at least 1 re-experiencing symptom and either 3 avoidance symptoms or 2 hyperarousal symptoms (Blanchard et al., 1995). Symptoms must have occurred at least once within the past month (frequency ≥ 1) and have caused a moderate amount of distress (intensity ≥ 2) (Weathers et al., 1999, 2001). Participants who completed the PCL questionnaire and had a minimum total score of 50 met the criteria for PTSD ($n = 3$), and those with scores from 39 to 49 met the criteria for partial PTSD ($n = 2$) (Hoge et al., 2008; Iversen et al., 2008; Renshaw, 2011; Weathers et al., 1993). Study participants with partial PTSD and PTSD were analyzed together ($n = 25$) to maintain statistical power and to examine broad group differences in PTSD neurocircuitry. The PTSD patients were not on medications at the time of the MEG exam. All had discontinued any psychotropic medications prior to the scan, and at least at 5-day wash-out.

We recruited thirty healthy volunteers (29 male, 1 female; mean [SD] age: 29.8 [6.4]) with no history of neurological or psychiatric disorders assessed by Structured Clinical Interview for DSM-IV. Among these healthy volunteers, 12 were active-duty military personnel and 18 were

civilians. Mean [SD] years of education were 13.4 [1.7]. There were no statistically significant differences in age or education between the healthy volunteer and PTSD groups.

Exclusion criteria for study participation were as follows: 1) other neurological, developmental or psychiatric disorders (e.g., brain tumor, stroke, epilepsy, Alzheimer's disease, or schizophrenia, bipolar disorder, history of learning disability, or lesions visible in structural MRI); 2) substance or alcohol abuse according to DSM-IV criteria within the 6 months prior to the study; 3) history of metabolic or other diseases known to affect the central nervous system (Dikmen et al., 1995); and 4) extensive metal dental hardware (e.g., braces and large metal dentures; fillings are permitted) or other metal objects in the head, neck, or face areas that cause non-removable MEG artifacts.

2.2. MEG data acquisition and signal pre-processing to remove artifacts

Resting-state MEG data (spontaneous recording for detecting MEG slow-wave signals) were collected at the UCSD MEG Center using the VectorView™ whole-head MEG system (Elekta-Neuromag, Helsinki, Finland) with 306 MEG channels. Participants sat inside a multi-layer magnetically-shielded room (IMEDCO-AG) (Cohen et al., 2002). Precautions were taken to ensure head stability; foam wedges were inserted between the participant's head and the inside of the unit, and a Velcro strap was placed under the participant's chin and anchored in superior and posterior axes. Head movement across different sessions was about 2–3 mm. MEG recording was divided into two 5-minute blocks with eyes closed, alternating with two 5-minute blocks with eyes open. In the eyes-closed condition, the participant was instructed to keep his/her eyes closed and empty his/her mind. In the eyes-open condition, the participant was instructed to fix his/her eyes on a fixation point and empty his/her mind. The order of blocks was counter-balanced between participants. Data were sampled at 1000 Hz and were run through a high-pass filter with a 0.1 Hz cut-off, and a low-pass filter with a 300 Hz cut-off. The filter associated with MEG data acquisition is a first-order time-domain filter with 3 dB around the cut-off points. Eye blinks, eye movements, and heart signals were monitored. Since the MEG eyes-open data were contaminated with eye-blinks in many participants, we focused on analyzing the eyes-closed data in the present study.

Substantial efforts were taken to help ensure that participants were alert during the MEG recordings. Participants were scheduled early in the day to avoid fatigue from performing daily activities. Prior to all of the study sessions, participants completed a questionnaire about the number of hours they slept the previous night, how rested they felt, and if there was any reason that they might not be attentive and perform to the best of their abilities (due to headache, pain, etc.). Sessions alternated between eyes-closed and eyes-open conditions, and eye blinking and movement were monitored. During MEG recording, participants were viewed on camera while technicians also monitored alpha band oscillations, which are consistently associated with tonic alertness (Oken et al., 2006).

Eyes-closed MEG data were first run through MaxFilter, also known as signal space separation (Song et al., 2008; Taulu et al., 2004a,b) to remove external sources of interference (e.g., magnetic artifacts due to metal objects, strong cardiac signals, environment noises), and to co-register the MEG data by removing the small head movements across the two 5-minute eyes-closed sessions. Next, residual artifacts due to eye movements and residual cardiac signals were removed using Independent Component Analysis using our customized version of ICALAB software (www.bsp.brain.riken.jp/ICALAB/).

2.3. Structural MRI, MEG-MRI registration, BEM forward calculation

Structural MRI of the participant's head was collected using a General Electric 1.5 T Excite MRI scanner. The acquisition contains a standard high-resolution anatomical volume with a resolution of $0.94 \times 0.94 \times 1.2\ mm^3$ using a T1-weighted 3D-IR-FSPGR pulse sequence. To co-register the MEG with MRI coordinate systems, three anatomical landmarks (i.e., left and right pre-auricular points, and nasion) were measured for each participant using the Probe Position Identification system (Polhemus, USA). By using MRILAB (Elekta/Neuromag) to identify the same three points on the participant's MR images, a transformation matrix involving both rotation and translation between the MEG and MR coordinate systems was generated. To increase the reliability of the MEG–MR co-registration, approximately 80 points on the scalp were digitized with the Polhemus system, in addition to the three landmarks, and those points were co-registered onto the scalp surface of the MR images. The T1-weighted images were also used to extract the brain volume and innermost skull surface (SEGLAB software developed by Elekta/Neuromag). Realistic Boundary Element Method (BEM) head model was used for MEG forward calculation (Huang et al., 2007; Mosher et al., 1999). The BEM mesh was constructed by tessellating the inner skull surface from the T1-weighted MRI into ~6000 triangular elements with ~5 mm size. A cubic source grid with 5 mm size was used for calculating the MEG gain (i.e., lead-field) matrix, which leads to a grid with ~10,000 nodes covering the whole brain. Other conventional MRI sequences typical for identifying structural lesions were also performed: 1) Axial T2*-weighted; 2) Axial fast spin-echo T2-weighted; and 3) Axial FLAIR. These conventional MRIs were carefully reviewed by a Board-certified neuroradiologist (R.R. Lee) to determine if the participant had visible lesions on MRI. Subjects with lesions visible in MRI were excluded from the study (see exclusion criteria).

2.4. MEG slow-wave source magnitude imaging using fast-VESTAL

The voxel-wise MEG source magnitude images were obtained using our recent high-resolution Fast-VESTAL MEG source imaging method (Huang et al., 2014a). This approach requires the sensor waveform covariance matrix. Here, the second 5-minute resting-state MEG sensor-waveform dataset was registered to the first 5-minute resting-state dataset using MaxFilter. The artifact-free, eyes-closed, resting-state MEG sensor-waveform datasets were divided into 2.5 s epochs. The data in each epoch were first DC-corrected and then run through band-pass filters for the following frequency bands: alpha band (8–12 Hz), beta band (15–30 Hz), gamma band (30–80 Hz), high-gamma band (80–150 Hz), and low-frequency band (1–7 Hz) that combined delta (1–4 Hz) and theta bands (4–7 Hz). Notch filters at 60 Hz and 120 Hz were applied to remove the power line signals and their second harmonics. Frequency-domain band-pass filter with zero phase-shift via discrete Fourier transform was used. At each end of the band-pass filter, the transition of the Hanning window in the filter was selected to be at 10% of the associated cut-off frequency.

Waveforms from all 306 sensors including 204 planar-gradiometers and 102 magnetometers were used in the analysis. For each frequency band, sensor-waveform covariance matrices were calculated for individual epochs after the band-pass filtering, then, the final sensor-waveform covariance matrix was obtained by averaging the covariance matrices across individual epochs for the 10-minute resting-state data. Using such a covariance matrix, MEG slow-wave source magnitude images that cover the whole brain were obtained for each participant following the Fast-VESTAL procedure (Huang et al., 2014a) for a given frequency band.

The brain volume is pre-divided into a grid of dipoles with P nodes. Let $\mathbf{R}$ be the $M \times M$ sensor-waveform MEG covariance matrix where M is the number of MEG sensors for a given frequency band (e.g., beta band) and time-window (e.g., length of an epoch); and $\mathbf{G}$ be the $M \times 2P$ gain (lead-field) matrix calculated from MEG forward modeling for the pre-defined source grid with P dipole locations, with each dipole location having two orthogonal orientations (i.e., θ and ϕ). In the spherical MEG forward head model, θ and ϕ represent the two tangential orientations for each dipole location, whereas in a realistic MEG forward model using the Boundary Element Method (BEM), the θ and ϕ-orientations

are obtained as the two dominant orientations from the singular-value decomposition (SVD) of the $M \times 3$ lead-field matrix for each dipole, as previously documented (Huang et al., 2006).

Eigen-value decomposition is performed for the sensor-waveform covariance matrix:

$$R = U_B \Sigma_B U_B^T = U_B S_B S_B^T U_B^T \quad (1)$$

where the diagonal elements in $\mathbf{S}_B$ are simply the square root (SQRT) of the corresponding eigenvalues of $\mathbf{R}$ which are the diagonal elements in $\mathbf{\Sigma}_B$. Next, SVD is performed for the gain matrix $\mathbf{G}$:

$$G = U_G S_G V_G^T \quad (2)$$

The dimensions for $\mathbf{U}_G$, $\mathbf{S}_G$, and $\mathbf{V}_G$ are $M \times M$, $M \times 2P$, and $2P \times 2P$, respectively. Following the procedure in (Huang et al., 2014a), a distributed source solution for Eq. 2 can be expressed as:

$$U_B S_B = U_G S_G V_G^T H \quad (3)$$

The $2P \times M$ matrix $\mathbf{H}$ is called the distributed *source spatial map* matrix. The goal of MEG inverse source imaging is to obtain $\mathbf{H}$ for given $\mathbf{R}$ in Eq. 3. However, Eq. 3 is under-determined, with the number of unknown variables in each column of $\mathbf{H} = [\mathbf{h}_1, \mathbf{h}_2, \ldots, \mathbf{h}_k, \ldots, \mathbf{h}_M]$ (i.e., $2P$) much larger than the number of measurements in each column of $\mathbf{U}_B\mathbf{S}_B = [s_1\mathbf{u}_1, s_2\mathbf{u}_2, \ldots, s_k\mathbf{u}_k, \ldots, s_M\mathbf{u}_M]$ (i.e., M), so additional constraint(s) are needed to obtain a unique solution for Eq. 3. Here, the number of signal (dominant) spatial modes k is usually much smaller than the number of MEG sensor measurements M. After multiplying from the left side with U_G^T, for individual dominant spatial modes of Eq. 3, Eq. 3 can be written as:

$$U_G^T u_i s_i = S_G V_G^T h_i, i = 1, 2, \ldots, k \quad (4)$$

where $i = 1,2,\ldots,k$ are the indices of spatial modes in sensor space.

By introducing additional minimum L1-norm constraints (Huang et al., 2014a) to Eq. 4, one can obtain the Fast-VESTAL solution for $\mathbf{h}_i$:

$$\min(w^T|h_i|), \text{ subject to constraints } S_G V_G^T h_i \cong U_G^T u_i s_i, i = 1, 2, \ldots, k \quad (5)$$

where the $2P \times 1$ vector $\mathbf{h}_i$ is the source imaging map associated with the dominant spatial mode vector $\mathbf{u}_i$ (dimension $M \times 1$) of the sensor-domain. In Eq. 5, $w = \sqrt{diag(V_G V_G^T)}$ is a $2P \times 1$ weighting vector chosen to remove potential bias towards grid nodes at the superficial layer (Huang et al., 2014a). After solving for $\mathbf{h}_i$ and hence $\mathbf{H}$ using Eq. 5, the Fast-VESTAL source imaging result can be obtained on the source grid as:

$$A = \sqrt{diag(HH^T)} \quad (6)$$

which is the $2P \times 1$ source magnitude value across grid nodes. The main feature of $\mathbf{A}$, the Fast-VESTAL-based distributed source solution, is that it is highly sparse, with many of its elements being either zero or close to zero, as a direct consequence of L1-norm minimization. An Objective Pre-whitening Method was applied to remove correlated environmental noise and objectively select the dominant eigen-modes (i.e., k) of sensor-waveform covariance matrix (Huang et al., 2014a).

2.5. Statistical analysis of MEG source magnitude images

Statistical analysis was performed separately for each frequency band. MEG source magnitude imaging volumes obtained from Fast-VESTAL that cover the whole brain from all healthy control and PTSD participants were first spatially smoothed using a Gaussian kernel with 3 mm full width half maximum (FWHM), and then co-registered to an MNI-152 brain-atlas template using FLIRT program in FSL software package (http://www.fmrib.ox.ac.uk/fsl/). For each voxel in the MNI space, the MEG source magnitude data were run through a logarithm transformation. A two-tailed t-test was performed to assess the group difference for each voxel of the brain volume in the MNI space. False discovery rate (FDR) was used to control the family-wise error (Benjamini and Hochberg, 1995) with $q < .05$. The above procedure was performed for each of the frequency bands separately.

2.6. Correlation with symptom scores in PTSD

For brain areas that showed group differences within a specific frequency band, regions of interest (ROIs) were obtained by grouping the voxels together. We were specifically interested in the ROIs that covered amygdala, vmPFC, OFC, precuneous, and dlPFC. Within each ROI, we performed a correlational analysis between MEG source magnitude and PTSD symptom score. The analyses were performed in the 20 participants with PTSD or partial PTSD as measured by CAPS Total score. The remaining 5 participants with PTSD or partial PTSD as measured by PCL were not included in this correlational analysis.

3. Results

3.1. Beta-band MEG source magnitude imaging results

Fig. 1 shows group differences between participants with PTSD and healthy volunteers in resting-state MEG source magnitude for the beta-band (15–30 Hz). Increased beta-band activity in PTSD (hyperactivity, PTSD > controls) was generated from bilateral amygdala and left anterior hippocampus (white arrows), left and right posterolateral OFC (magenta arrows), several regions within the right insular cortex, bilateral middle temporal gyri, right posterior cingulate cortex (PCC, brown arrow), bilateral junctions of PCC and lingual gyri, and left occipito-temporal–parietal junction. In addition, Fig. 1 shows decreased beta-band activity in PTSD (hypoactivity, PTSD < controls) from vmPFC (green arrows) including rostral anterior cingulate cortex (rACC) and medial OFC, bilateral FPs (more R than L), bilateral caudate, bilateral dlPFC (more R than L), right superior frontal gyrus, mid-line supplementary motor areas (SMA), right anterior aspect of superior temporal gyrus, bilateral precuneous cortices, and bilateral sensorimotor cortices (more R than L). For a region, an asymmetry is reported when one hemisphere has twice or more voxels being significant than the equivalent region in the opposite hemisphere.

3.2. Gamma and high-gamma-bands MEG source magnitude imaging results

The upper panel of Fig. 2 shows increased gamma-band (30–80 Hz) activity in PTSD compared to the healthy control group that was generated from left and right posterolateral OFC (magenta arrows, more L than R), bilateral dmPFC including the dorsal paracingulate cortices and dorsal anterior cingulate cortex (dACC) (more L than R), several regions within the bilateral insular cortices, bilateral occipito-temporal–parietal junctions (more L than R), bilateral temporal–occipital fusiform cortices (more R than L), left occipital fusiform gyrus and right lingual gyrus, and right dorsomedial occipital cortex. The upper panel of Fig. 2 also shows decreased gamma-band activity in PTSD compared to the control group from vmPFC (green arrows) including rACC and medial OFC, bilateral FPs (more R than L), right dlPFC, mid-line SMA, and right sensorimotor cortices.

The lower panel of Fig. 2 shows increased high-gamma-band (80–150 Hz) activity in PTSD from left amygdala and hippocampus (white arrows), left posterolateral OFC (magenta arrows), right dACC, left FP, several regions within the bilateral insular cortices, bilateral occipito-temporal–parietal junctions (more L than R), and right dorsomedial occipital cortex. The lower panel of Fig. 2 also shows

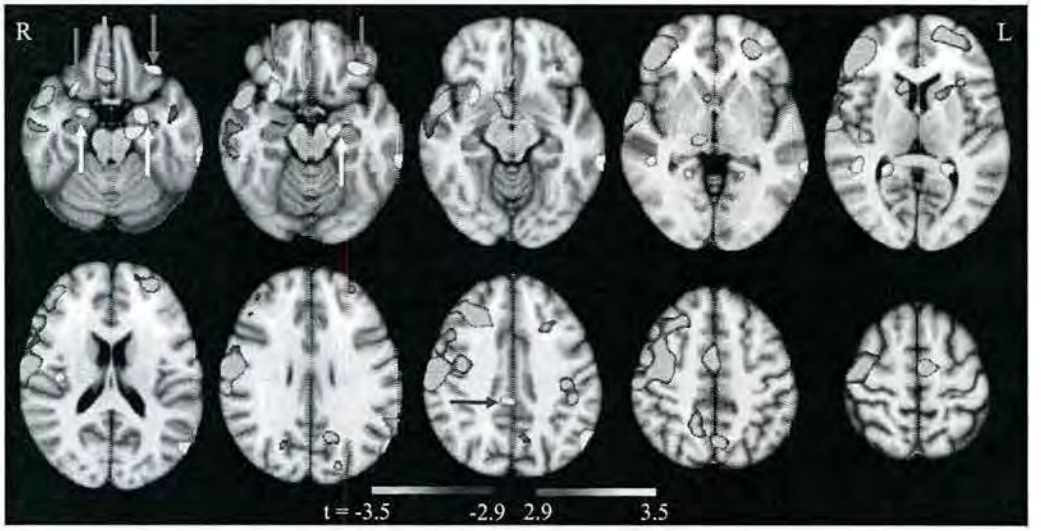

Fig. 1. Abnormal beta band (15–30 Hz) MEG activity in PTSD. Red-yellow color scale indicates increased (hyper-) activity in PTSD over health controls, whereas blue-cyan color scale indicates decreased (hypo-) activity in PTSD over health controls. White arrows: amygdala and hippocampus activity. Green arrows: vmPFC activity. Magenta arrows: posterolateral OFC activity. Brown arrow: PCC activity. The t-threshold of 2.9 is associated with FDR corrected $p < .05$.

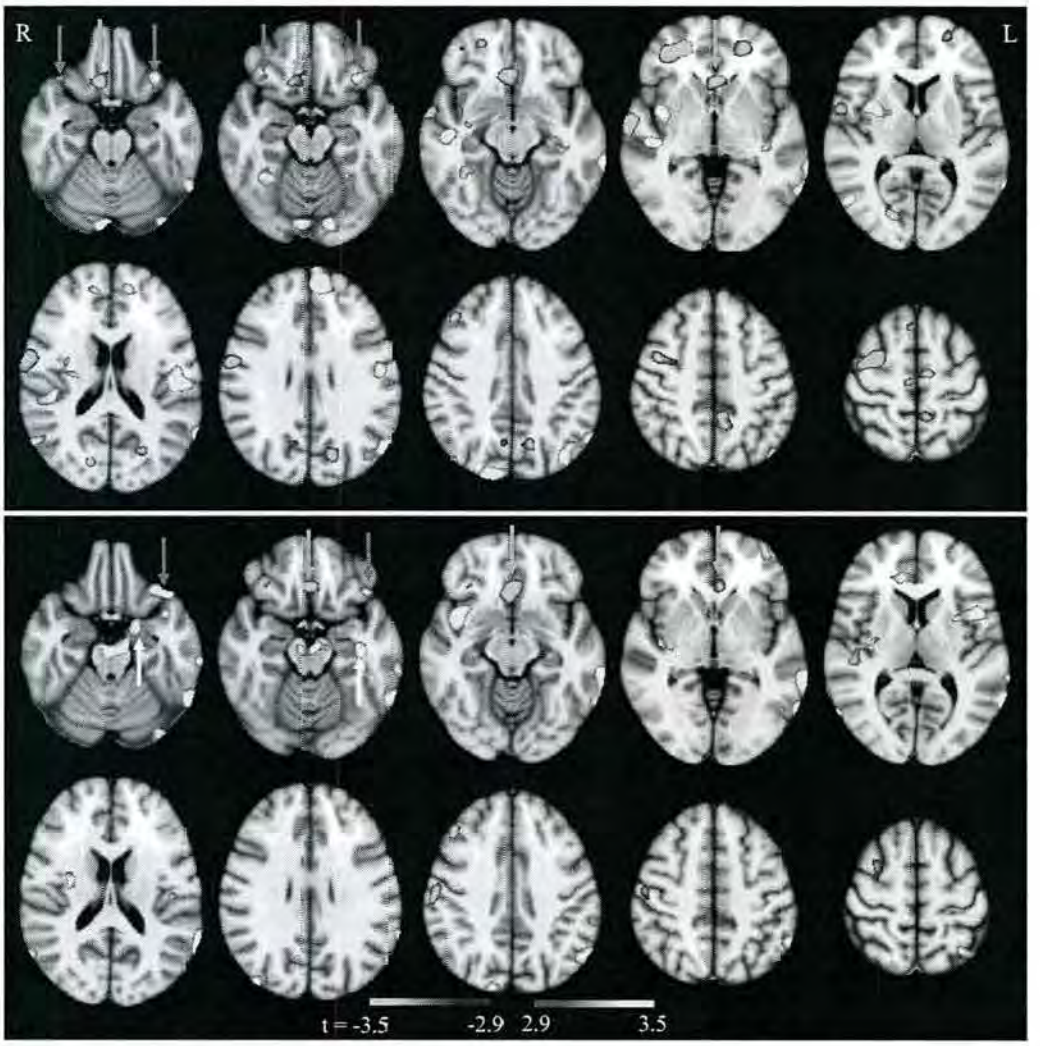

Fig. 2. Top panel: abnormal gamma band (30–80 Hz) MEG activity in PTSD; bottom panel: abnormal high-gamma band (80–150 Hz) MEG activity in PTSD. Red-yellow color scale indicates increased (hyper-) activity in PTSD over health controls, whereas blue-cyan color scale indicates decreased (hypo-) activity in PTSD over health controls. White arrows: amygdala and hippocampus activity. Green arrows: vmPFC activity. Magenta arrows: posterolateral OFC activity. The t-threshold of 2.9 is associated with FDR corrected $p < .05$.

decreased high-gamma-band activity in PTSD from mid-line vmPFC (green arrows) including rACC and medial OFC, right dlPFC, and right sensorimotor cortices.

3.3. Alpha and low-frequency bands MEG source magnitude imaging results

Although PTSD was associated with both hyper- and hypoactivity in the beta, gamma, and high-gamma bands, alpha band MEG activity was largely hypoactive in PTSD when compared with the healthy volunteers. The upper panel of Fig. 3 shows significantly decreased alpha-band activity in PTSD generated from bilateral FPs, bilateral dlPFC (more R than L), right superior frontal gyrus, bilateral anterior aspects of superior temporal gyri (more R than L), bilateral precuneous cortices, and bilateral sensorimotor cortices (more R than L). In contrast, only the left occipito-temporal–parietal junction showed increased alpha-band activity in PTSD.

PTSD was also strongly associated with hypoactivity in the low-frequency band compared to the healthy volunteers. The lower panel of Fig. 3 shows significantly decreased alpha-band activity in PTSD generated from bilateral FPs, bilateral dlPFC (more R than L), bilateral anterior aspects of superior temporal gyri (more R than L), bilateral precuneous cortices, and bilateral sensorimotor cortices (more R than L). Similar to the patterns observed in the alpha band, only the left occipito-temporal–parietal junction showed increased low-frequency-band activity in PTSD.

3.4. Results of MEG source magnitude correlating with PTSD symptoms

Positive correlations between resting-state MEG activity and CAPS Total symptom scores were found in left amygdala (beta band, $r = +0.51$, $p < .05$) and left posterolateral OFC (also in beta band, $r = +0.55$, $p < .05$), indicating the stronger the resting-state MEG activity in these areas, the more severe the PTSD symptoms. In addition, negative correlations between resting-state MEG activity and total CAPS symptom scores were found in midline vmPFC (beta band, $r = -0.58$, $p < .01$; gamma band, $r = -0.63$, $p < .01$; and high-gamma band, $r = -0.60$, $p < .01$), and midline precuneous (alpha band, $r = -0.48$, $p < .05$), indicating the weaker the resting-state MEG activity in these areas, the more severe the PTSD symptoms.

4. Discussion

For the first time to our knowledge, the present study shows that individuals with PTSD have abnormal electromagnetic activity that can be directly *imaged* by resting-state MEG source imaging technique for all

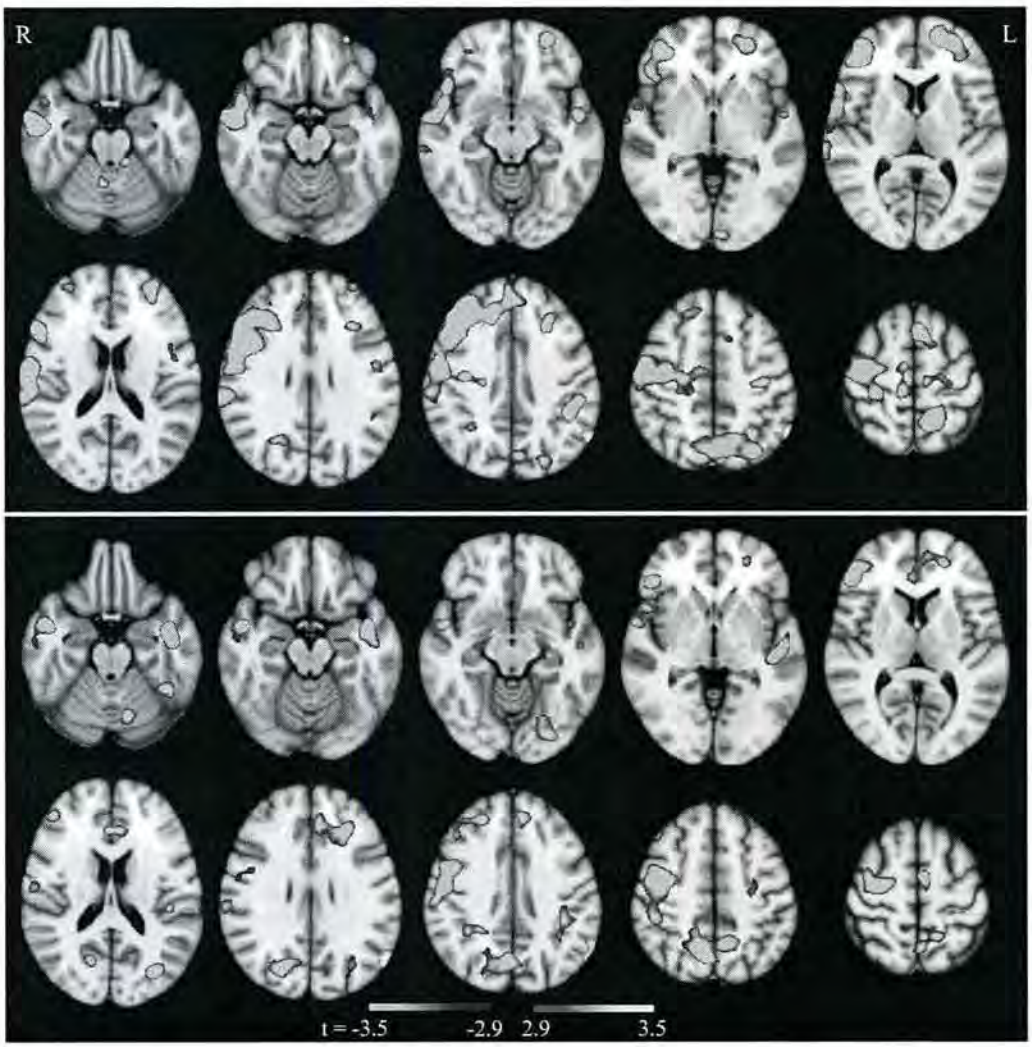

Fig. 3. Top panel: abnormal alpha band (8–12 Hz) MEG activity in PTSD; bottom panel: abnormal low-frequency band (1–7 Hz) MEG activity in PTSD. The t-threshold of 2.9 is associated with FDR corrected $p < .05$.

frequency bands. PTSD was associated with: 1) MEG hyperactivity from amygdala, hippocampus, posterolateral OFC, dmPFC, insular cortex, and PCC in high frequency bands (i.e., beta, gamma, and high gamma bands); 2) MEG hypoactivity from vmPFC, FP, and dlPFC in the high frequency bands; 3) extensive MEG hypoactivity from dlPFC, FP, anterior temporal lobes, precuneous cortex, and sensorimotor cortex in alpha and low-frequency bands, with dlPFC and sensorimotor cortex hypoactivity more prominent in right versus left hemispheres; and 4) resting-state MEG activity in left amygdala and posterolateral OFC positively correlated with PTSD symptom scores, whereas MEG activity in vmPFC and precuneous correlated negatively with the PTSD symptoms.

Neuronal activity from different frequency bands is considered to reflect different neuronal mechanisms. Thalamo-cortical interactions are essential for alpha rhythms, and normal alpha-band activity is associated with functional inhibition; specifically, increased alpha-band power in a brain area was linked to reduced functional connectivity with other brain areas (De Munck et al., 2009; Hindriks and van Putten, 2013; Scheeringa et al., 2012). Activity in the beta band is associated with communication between remote brain structures, whereas gamma synchrony promotes local computations (Kopell et al., 2000; Singer, 1999). Although the gamma band electromagnetic signals are generated locally, non-local brain areas can still show significant functional connectivity as measured by coherence related to the gamma band signals. Using combined electrophysiological and fMRI measurements, studies in both human and animals showed that gamma-band power exhibits spatial coherence over long timescales with the strongest coherence between functionally related areas that are not necessarily local (He et al., 2008; Nir et al., 2008; Scholvinck et al., 2010, 2013; Shmuel and Leopold, 2008). Unlike alpha-band activity, beta and gamma-band activity does not necessarily have to involve thalamus. Theta-band signals have been reported in previous EEG studies, although these signals were predominantly task-activated (e.g., problem solving) (Mizuki et al., 1980, 1984, 1992; Niedermeyer and Lopes da Silva, 2005; Takahashi et al., 1997). Increased low-frequency brain rhythms in delta band were often seen in individuals with neurological disorders, e.g. epilepsy and traumatic brain injury (Baayen et al., 2003; de Jongh et al., 2003; Decker and Knott, 1972; Huang et al., 2009, 2012, 2014b; Lewine et al., 1999; Lewine and Orrison, 1995; Nagata et al., 1985; Vieth et al., 1996). When examining the mechanism of abnormal delta rhythms, electrophysiological studies in animals show that abnormal delta activity is from gray matter neurons that have experienced deafferentation due to neurological injuries in underlying white matter, resulting from axonal injury or blockage/limitation in the cholinergic pathways (Ball et al., 1977; Gloor et al., 1977; Schaul et al., 1978; Schaul, 1998).

4.1. MEG findings in amygdala and hippocampus

Individuals with PTSD showed amygdala hyperactivity. Our MEG findings are consistent with previous PET and fMRI findings of hyperresponsive amygdala activity in PTSD (Rauch et al., 1998, 2006), which is one of the most robust functional neuroimaging findings in PTSD (Hughes and Shin, 2011). The amygdala is involved in processing threat-related stimuli (Davis and Whalen, 2001; Morris et al., 1998; Whalen et al., 1998, 2001) and is necessary for fear conditioning (Davis and Whalen, 2001; LeDoux, 2000; Shin et al., 2006). Moreover, the amygdala is a key component in the neurocircuitry model of PTSD (Rauch et al., 2006). The present MEG study shows that the amygdala hyperactivity in PTSD can also be detected using electromagnetic source imaging measures, which increases the confidence in our MEG technique for detecting new abnormalities in PTSD. Furthermore, we demonstrate that amygdala hyperactivity was only observable in the high frequency bands (i.e., beta and high-gamma bands). In addition, the MEG hyperactivity in PTSD from left hippocampus in beta and high-gamma bands is also consistent with the current PTSD neurocircuitry model (Rauch et al., 2006), although the findings from previous PET and fMRI in this region have been mixed (Hughes and Shin, 2011).

4.2. MEG findings in dmPFC and insula

The MEG gamma-band hyperactivity from dmPFC, including the dACC, in PTSD was also consistent with prior PET and fMRI findings (Bremner et al., 1999; Felmingham et al., 2009; Pannu et al., 2009; Shin et al., 2001, 2007, 2011). The dmPFC, including the dACC, is thought to play an important role in a variety of cognitive processes such as performance monitoring, response selection, error detection, and decision making (Shin et al., 2011). In addition, PTSD was associated with increased MEG insular activity. Our findings are consistent with studies that used trauma-event-script-driven imagery with SPECT (Lindauer et al., 2008) and fMRI (Lanius et al., 2007), as well as with studies that used emotional and trauma-unrelated stimuli with PET and fMRI (Hughes and Shin, 2011). Painful stimuli have also been shown to increase insular activity in PTSD (Geuze et al., 2007; Strigo et al., 2010). The insular cortex processes information about the body's internal state and contributes to the autonomic component of the overall pain response. It has been suggested that the insular cortex integrates the sensory, affective, and cognitive components necessary for normal responses to pain (Kandel et al., 2000). Abnormal insular activity in PTSD may reflect a deficit in integrating these components, thereby contributing to an abnormal pain response (Nagai et al., 2007).

4.3. MEG findings in vmPFC

MEG hypoactivity from vmPFC in PTSD was consistent with findings from PET and fMRI studies (Hughes and Shin, 2011; Rauch et al., 1998, 2006). Hyporesponsive vmPFC is another key component in the current neurocircuitry model of PTSD (Rauch et al., 2006), which suggests that hyporesponsive vmPFC fails to suppress the amygdala (Rauch et al., 2006; Shin et al., 2006). The vmPFC is connected to and receives input from the ventral tegmental area, amygdala, temporal lobe, olfactory system, and dorsomedial thalamus. In turn, vmPFC sends signals to amygdala, temporal lobe, lateral hypothalamus, hippocampal formation, cingulate cortex, and certain other regions of the prefrontal cortex (Carlson, 2013). In the present study, hypoactivity in vmPFC associated with PTSD was evident in beta, gamma, and high-gamma bands, but not the lower frequency bands. These findings suggest that the beta- and gamma-band interactions between vmPFC and amygdala may not involve thalamus, as evidenced by the lack of group differences in vmPFC in thalamus-dependent alpha band activity (Hindriks and van Putten, 2013).

4.4. Resting-state MEG versus resting-state fMRI

We used a resting-state protocol that is insensitive to stimulus features and participant performance. Furthermore, we focused on examining MEG source-magnitude images rather than functional connectivity (Jin et al., 2013). Our protocol was similar to a recent resting-state fMRI study of combat-related PTSD that used a magnitude imaging approach (Yan et al., 2013). Our findings are consistent with Yan and colleagues, who also found that individuals with PTSD showed increased activity in amygdala, insular cortex, and OFC, as well as decreased activity in dlPFC, superior frontal gyrus, and precuneous cortex. Despite these similarities, participants with PTSD in the present study showed *decreased* MEG activity in vmPFC (beta, gamma, and high-gamma bands, see Figs. 1 and 2) and bilateral FP areas (Figs. 1–3), whereas Yan and colleagues, in their fMRI study showed *increased* activity in a similar region (Yan et al., 2013). Although it is known that the fMRI measurements in ventral frontal lobe areas are challenging to obtain due to signal loss, imaging distortion, and susceptibility artifacts (Czervionke et al., 1988; Domsch et al., 2013), the exact reason of the decreased MEG versus increased fMRI resting-state activity in vmPFC is unknown.

Overall, the beta-band MEG source imaging maps are similar to the fMRI maps of Yan and colleagues, except for the activity in vmPFC. Such a degree of similarity across two different imaging modalities (i.e., electromagnetic measures from MEG and hemodynamic measures from fMRI) is likely due to beta-band synchronization over long conduction delays, which corresponds to signals traveling a significant distance across brain regions. Electrophysiological studies of the rat hippocampus have shown that the beta rhythm allows neuronal synchrony at large time delays, while the gamma band allows such synchrony at short delays. Thus, beta synchrony promotes communication between remote structures, whereas gamma synchrony promotes local computations (Kopell et al., 2000; Singer, 1999). Interestingly, more recent work in identifying MEG correlates of fMRI resting-state networks has found that power fluctuations in the beta band produce spatial networks very similar to fMRI resting-state networks (Brookes et al., 2011b). Our findings suggest that abnormal beta-band neuronal activity in PTSD is likely a candidate for the abnormal fMRI signal observed by Yan and colleagues (Yan et al., 2013).

The consistent finding of decreases of resting-state activity in precuneous and dlPFC associated with PTSD in the present MEG study (in beta, alpha, and low-frequency bands) and in the fMRI study by Yan et al. (Yan et al., 2013) highlight the contribution of these regions in PTSD neurocircuitry. The precuneous is a key region of the "default-mode network (DMN)" in resting brain which has been detected by fMRI (Fransson and Marrelec, 2008) and MEG (Brookes et al., 2011b). Furthermore, the precuneous plays a pivotal role in how intrinsic activity is mediated throughout the DMN, and helps sustain a sense of self-consciousness in self-referential mental thoughts during rest (Cavanna and Trimble, 2006; Fransson and Marrelec, 2008). Non-trauma related words elicit decreased precuneous fMRI activity in PTSD, and the decrease in precuneous activity is correlated with CAPS scores (Geuze et al., 2008). Dissociative symptoms of patients with PTSD may play a role in the decreased activation of precuneous (Geuze et al., 2008). The dlPFC is a key region for a variety of executive brain functions such as working memory, attention, and other executive functions. It facilitates goal-directed behavior through indirect modulation of the amygdala response to threat, possibly through connections with the temporal cortex (Bishop, 2008; Gold et al., 2014; Mitchell, 2011). In the present study, MEG hyperactivity in both right dlPFC and anterior temporal lobe in alpha, beta, gamma, and low-frequency (Figs. 1–3) is consistent with the modulation deficit of the dlPFC–anterior temporal-amygdala pathway in PTSD. Using a task involving cognitive regulation of negative affect via reappraisal, Rabinak and colleagues found that PTSD patients engaged the dlPFC during cognitive reappraisal, albeit to a lesser extent than the control participants (Rabinak et al., 2014). In a longitudinal cortical thickness study, individuals with PTSD showed greater dlPFC thickness in a follow-up exam about 1 year after the trauma than in the acute exam, and greater dlPFC thickness was associated with greater PTSD symptom reductions and better recovery (Lyoo et al., 2011). On the other hand, healthy volunteers showed greater dlPFC activation and increased amygdala connectivity to threats compared to non-threat condition (Gold et al., 2014). Elevated activity in dlPFC was also observed in PTSD during a maintenance period of working memory in an fMRI test (Moores et al., 2008). Future functional imaging studies of PTSD are needed to examine the association between resting-state dlPFC activity and dlPFC responses to different types of working memory and/or attention stimuli.

In an event related potential (ERP) study of combat veterans with PTSD after mild TBI by Shu and colleagues, PCC and precuneous areas exhibit greater ERPs evoked by emotional facial stimuli (Shu et al., 2014). In the present study, PCC also showed hyperactivity in the beta-band resting-state MEG source image (brown arrow in Fig. 1), a finding consistent with the above ERP study. However, the hypoactive precuneous is seen in our resting-state MEG source image across many frequency bands, also observed in resting-state fMRI by Yan and colleagues (Yan et al., 2013), seems to be different from the greater ERPs in precuneous found by Shu and colleagues using emotional stimuli. Additional studies are needed to directly examine the association between resting-state electromagnetic signal and evoked responses, as well as the impact of mild TBI on PTSD.

4.5. MEG findings in OFC

Another interesting finding from the present study is the increased activity from the posterolateral OFC areas in beta, gamma, and high gamma bands. Our finding is consistent with fMRI findings of increased resting-state activity in PTSD (Yan et al., 2013). The OFC is closely connected to the limbic system, especially the amygdala, and is sometimes regarded as part of the expanded limbic system (Nauta, 1979). While regions known to be part of the existing neurocircuitry model of PTSD (i.e., amygdala, vmPFC, and insular cortex) have been studied extensively (Rauch et al., 1998, 2006; Shin et al., 2006), the role of the posterolateral OFC in PTSD is unclear and should be examined further. Based on our MEG findings, posterolateral OFC activity increased with PTSD symptom severity, thus OFC and its interactions with the amygdala may be added to the existing neurocircuitry model of PTSD. This idea is supported by studies that show that OFC has direct anatomical projections to the amygdala and hippocampus via the uncinate fasciculus in humans (Bach et al., 2011; Talairach and Tournoux, 1988) as well as in non-human primates (Carmichael and Price, 1995). It was also shown that such projections were abnormal in some psychiatric disorders such as conduct disorder (Passamonti et al., 2012), bipolar disorder (Benedetti et al., 2011), and schizophrenia (Jackowski et al., 2012). Further studies are needed to confirm whether disrupted interactions between OFC-amygdala may be implicated in PTSD.

4.6. Decreased MEG alpha-band activity in PTSD

Individuals with PTSD showed extensive MEG alpha-band hypoactivity from dlPFC, FP, anterior temporal lobes, precuneous cortex, and sensorimotor cortex. Neuronal modeling studies showed that thalamo-cortical interactions are essential to the generation of alpha rhythms (Freyer et al., 2011; Hindriks and van Putten, 2013; Lopes da Silva et al., 1997). Combined EEG and fMRI studies have also shown that increased alpha-band power in a brain area is associated with reduced functional connectivity with other brain areas, suggesting that alpha-band activity is associated with functional inhibition (De Munck et al., 2009; Scheeringa et al., 2012). The observed MEG alpha-band hypoactivity may suggest a deficit in thalamo-cortical interactions, which possibly leads to reduced functional inhibition in the above cortical areas in PTSD. In general, a normal amount of alpha activity is preferred in the resting-state, and reduced alpha-band power has also been observed in individuals with Alzheimer's disease (Babiloni et al., 2013; Tartaglione et al., 2012), and schizophrenia (Hinkley et al., 2011; Sponheim et al., 2000).

4.7. MEG source imaging with fast-VESTAL

Our method plays an essential role in obtaining the source magnitude images for the neurocircuitry in PTSD (Figs. 1–3). It was shown that Fast-VESTAL can effectively create resting-state MEG source images that are highly consistent with known neurophysiology findings (Huang et al., 2014a). We have shown that for resting-state MEG signal, the source magnitude images obtained using a beamformer technique (a popular MEG source analysis method) are less consistent with neurophysiology findings (Huang et al., 2014a). This is likely due to beamformer's intrinsic limitation which assumes the neuronal sources are uncorrelated (Robinson and Vrba, 1999; Sekihara et al., 2001; Van Veen et al., 1997), a questionable assumption when dealing with resting-state MEG signals.

In the present study, we focus on MEG source magnitude images in PTSD. No results were presented regarding the MEG-based connectivity

analyses. This is because we are in the process of finalizing the Fast-VESTAL based voxel-wise MEG connectivity method (Huang et al., in preparation). Although MEG-based connectivity study is a hot topic in literature, with most published approaches used Beamformer or minimum L2-norm based techniques to obtain the source time-courses (Brookes et al., 2011a,b, 2012; Ghuman et al., 2011; Gramfort et al., 2014). It was known that source time-courses obtain by Beamformer are distorted when multiple correlated neuronal sources contribute to the sensor-waveforms even at noiseless cases (Huang et al., 2014a), and across-talk between source time-courses from minimum L2-norm approaches can also be a serious issue. However, even though many researchers were aware of the issues associated with distorted source time-courses, the impact on a variety of connectivity measures using the distorted source time-courses has not been examined thoroughly in resting-state data, at least to our knowledge. Before we publish our method for Fast-VESTAL based connectivity analysis, we believe that it would be beyond the scope of the present study to include MEG connectivity results for the PTSD population.

There are several limitations of the present study that warrant consideration. First, the spatial resolution and localization accuracy of MEG are expected to be limited for amygdala, hippocampus, and vmPFC, which may explain some minor location discrepancies between our findings and those of previous fMRI or PET studies. Second, although we acquired resting-state MEG signal in the eyes-closed condition, eye-movements and micro-eye-blinks may be confounding factors. Although we pre-processed the MEG data through both MaxFilter and ICA to remove the eye-movement and micro-eye-blinks, the impact of residual eye-activity-related artifacts may not be totally negligible. Third, despite substantial efforts to ensure and monitor alertness during the eyes-closed condition (see Materials and methods), drowsiness may still have had an impact on the MEG recording. Fourth, since the active-duty and veteran PTSD patients are mostly males, the present study is dominated by male subjects, with just one woman in each of the two groups.

Despite these limitations, the present study showed that our MEG source imaging technique revealed new abnormalities in the resting-state electromagnetic signals from PTSD neurocircuitry. Abnormal resting-state electromagnetic signals in PTSD neurocircuitry can be effectively imaged by MEG source imaging technique for different frequency bands. In high frequency bands (i.e., beta, gamma, and high gamma bands), PTSD was associated with 1) MEG hyperactivity from amygdala, hippocampus, posterolateral OFC, dmPFC, and insular cortex; and 2) MEG hypoactivity from vmPFC, FP, and dlPFC. In alpha and low-frequency bands, PTSD was associated with extensive MEG hypoactivity from dlPFC, FP, anterior temporal lobes, precuneous cortex, and sensori-motor cortex. Lastly, PTSD symptom scores correlated positively with resting-state MEG activity in left amygdala and posterolateral OFC and negatively with MEG activity in vmPFC and precuneous. Particularly, posterolateral OFC and precuneous may play important roles in the PTSD neurocircuitry model.

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Heart rate variability characteristics in a large group of active-duty Marines and relationship to posttraumatic stress

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Abstract

Objective—Heart rate variability (HRV), thought to reflect autonomic nervous system function, is lowered in conditions such as posttraumatic stress disorder (PTSD). The potential confounding effects of traumatic brain injury (TBI) and depression in the relationship between HRV and PTSD have not been elucidated in a large cohort of military service members. Here we describe HRV associations with stress disorder symptoms in a large study of Marines, while accounting for well-known covariates of HRV and PTSD including TBI and depression.

Methods—Four battalions of male active-duty Marines (N=2430) were assessed 1-2 months prior to a combat deployment. HRV was measured during 5 minutes of rest. Depression and PTSD were assessed using the Beck Depression Inventory and Clinician Administered PTSD scale respectively.

Results—After accounting for covariates including TBI, a regression indicated that lower levels of high frequency (HF) HRV were associated with a diagnosis of PTSD (beta = -.20, p=.035). Depression and PTSD severity were correlated (r= .49, p <.001), however participants with PTSD but relatively low depression scores exhibited reduced HF compared to controls (p=.012). Marines with deployment experience (n=1254) had lower HRV than those with no experience (p = .033).

Conclusions—This cross-sectional analysis of a large cohort supports associations between PTSD and reduced HRV when accounting for TBI and depression symptoms. Future post-deployment assessments will be used to determine whether pre-deployment HRV can predict vulnerability and resilience to the serious psychological and physiological consequences of combat exposure.

Conflict of Interest and Source of Funding
The authors have no conflict of interest to declare.

Keywords

sympathetic nervous system; PTSD; vagal tone; combat; depression; parasympathetic

Introduction

Heart rate variability (HRV), the quantitative assessment of variation in heartbeat intervals, can be used to detect alterations in autonomic nervous system (ANS) function (1). Heart rate is in part determined by influences on the sinoatrial node (SA) pacemaker, which is modulated by both the parasympathetic and sympathetic branches of the ANS (2). Spectral analysis of inter-beat intervals is used to derive the high-frequency (HF) peak of HRV, which is thought to reflect parasympathetic or vagal tone, though controversy exists about the sensitivity and specificity of widely-used HRV measures (3, 4).

HRV and regulation of the autonomic nervous system have been suggested to be useful in understanding cardiovascular and other health risks (4, 5). Decreased HRV has been associated with pathophysiology, psychopathology, and increased mortality (2, 6). Previous studies have reported lower HRV in psychiatric disorders such as schizophrenia, depression, bipolar disorder, panic disorder (7-10), and posttraumatic stress disorder (PTSD) (11-13). Accurate assessment of HRV can be done in a rapid (5-minute) period of time (14) using relatively non-obtrusive instrumentation; thus this physiological index has been used to study populations that may be at high risk for disrupted ANS functioning and the associated health complications.

One such high-risk group is represented by United States military service members who are deployed to combat situations such as Operation Enduring Freedom (Afghanistan post 2001), Operation Iraqi Freedom, or Operation New Dawn (OEF/OIF/OND). Since the onset of these conflicts, the prevalence of PTSD in returning veterans has been reported at 13-15% (15, 16). Prior studies have shown an association between PTSD and lower HRV (8, 11-13). PTSD is strongly associated with the occurrence of a traumatic brain injury (TBI) in OEF/OIF war veterans (17); furthermore TBI in and of itself has been related to lower HRV (18). Thus an unanswered question is whether HRV is associated with PTSD even when TBI is accounted for, in a population at elevated risk for both TBI and PTSD. Depression is also an important factor in understanding the relationship between trauma and HRV as illustrated by several studies on civilian trauma survivors (19, 20). Unknown, however, is the potential role of depressive symptoms in influencing the relationship between HRV and PTSD in military service members who are at high risk for both psychiatric conditions. Military personnel may constitute a unique population with respect to trauma and autonomic function due to a number of factors including prevalence of TBI, physical fitness, repeated exposure to severe combat-related trauma, and other features distinct to military service members.

The current study's objective was two-fold: 1) Assess the relationship between PTSD and HRV in a large group of active-duty Marines while accounting for important covariates of PTSD and HRV such as TBI and depression; and 2) Introduce the methodology used to assess HRV in this large participant group and assess the influence of potential covariates such as age (21, 22), ancestry (23), and use of nicotine (24) and caffeine (25, 26). This latter

goal is in the service of studying HRV changes in the longitudinal portion of this study, i.e., after these service members return from deployment. The quantification of ancestry using genetic markers versus relying on participant self-report is a novel approach to replicating previous associations between HRV and ethnicity (23).

We hypothesized that lower HRV as quantified by the HF component would be associated with a PTSD diagnosis even when TBI was accounted for. We further hypothesized, based upon previous findings in civilian trauma survivors (20), that the co-occurrence of depressive symptoms and PTSD would result in lower HRV than PTSD alone.

Methods

Participants

Participants were active-duty Marines who were tested approximately one month prior to deployment to OEF, OIF, or OND as part of the Marine Resiliency Study (MRS), a prospective longitudinal study whose objective is to examine markers of risk and resilience to effects of combat stress in active-duty Marines. Four unique Infantry battalions (cohorts) of Marines were tested between July 2008 and August 2010 at one of two bases: Marine Corps Air Ground Combat Center (MCAGCC) in Twenty-nine Palms, CA (Cohorts 1, 2, and 3) and Camp Pendleton, CA (Cohort 4). See Table 1 for individual cohort sizes. This study was approved by the institutional review boards of the Veteran's Administration San Diego Health System IRB, the University of California San Diego, and the Naval Health Research Center.

All active-duty Marines who were planning to deploy with their units were considered for inclusion into the study. Females were not included, since female Marines are not currently part of Infantry battalions. Since the US Marine Corps maintains specific guidelines that prohibit the severely mentally ill (i.e., Schizophrenia, Psychotic Disorder, Bipolar Disorder) from active-duty service, Marines with these pre-existing mental illness conditions were not included in the study.

The overall demographic composition of Marines in the MRS has been previously reported (27). Age, ancestry, and other covariate data for the participants are found in Table 2. 64.8% of participants reported graduating high school or receiving a GED, 32.1% reported some college or a college degree, and 0.4% reported a masters or doctoral-level degree. 61.8% of participants reported their marital status as never married, 36.1% reported being married, and 2.1% reported being separated or divorced. 68.3% had a junior enlisted rank (E1-E3), 28.9% were non-commissioned officers (E4-E9), and 2.6% were commissioned or warrant officers. Participants reported an average of 36.2 months (standard deviation=34.6) of military service. 48.2% reported at least one prior deployment.

Procedure

All participating Marines provided voluntary written informed consent. Marines were informed about confidentiality relating to research data, and research records were rigorously protected. The entire MRS test battery was approximately four hours in duration and included a comprehensive evaluation of demographic information, history, and current

symptoms with respect to military service, drug, alcohol and tobacco use, psychiatric conditions, head injuries, and psychological trauma (27). Height and weight were measured and blood samples were taken for genetic association studies.

For the assessment of HRV, participants were seated in quiet rooms. A finger photoplethysmograph (PPG, Pasco Scientific, Roseville, CA) was placed on the nail of the right fifth finger which rested on a desk. PPG is an optical technique used to detect beat-to-beat blood volume changes, for example as a result of pulse, in microvascular tissue. Fluctuations in the blood volume of the finger are directly related to the activity of the heart, thus the interval between peaks in the PPG signal, known as the PP interval, is considered a reasonably accurate reflection of the RR interval (28). PPG has been shown to be a sensitive and reliable peripheral instrument for the capture of cardiac activity (29), for example it is highly correlated with waveforms from simultaneous ECG recordings (30, 31). Frequency and time-domain measures of HRV derived from PPG were not significantly different from those derived by a simultaneous two-lead ECG recording (28). PPG was sampled at the rate of 1000 Hz. Using an oscilloscope display and amplification of the PPG signal (San Diego Instruments), the examiner ensured that the PPG was adequately capturing the heart beat waveforms without cutting off the peak of the R wave. The position of the PPG was adjusted until a visually clear heart-beat signal was obtained and each Marine was asked to keep his hand relatively motionless during the 5 min recording. Participants were asked to sit comfortably and direct their attention to a computer monitor where they were entertained with simple visual puzzles (e.g. locating hidden images in a photograph). The images were selected to be neutral and minimally affectively arousing or stress-inducing (e.g., dolphins, frogs). They were told that they did not need to memorize anything and that they would not be tested on the images afterward. The purpose of the hidden image task was to maximize the likelihood that participants remained stationary, awake, and alert for the duration of the recording. The images changed every 60 seconds (thus outside of the bandwidth for both LF and HF frequency ranges—see below) and the order of presentation of the images was the same for all participants. The 5-minute HRV recording session was simultaneous with the 5-minute acclimation period which is standardly used immediately prior to a session of eyeblink startle and prepulse inhibition measurement (32, 33). Thus, during the 5-minute PPG recording session participants wore headphones which delivered continuous broad-band noise at a decibel level of 70 dB, which is a standard level for an acclimation period prior to an eyeblink startle session. Participants were also prepared for electromyography (EMG) of the orbicularis oculi muscle recordings via the placement of two skin electrodes near the left eye for the purpose of subsequently assessing the eyeblink startle response and sensorimotor inhibition after the heart rate recordings presented here were completed (EMG data will be reported elsewhere). Only the constant 70 dB background noise was delivered during the 5-minute HRV recording.

Data Processing

Data files (one file per 5-minute session) from the PPG were extracted and processed through a multi-step procedure to generate HRV variables:

1. The systolic peaks of the PPG signal were identified and a tachogram representing intervals between heartbeats (the PP interval) was generated by measuring the time difference between successive peaks.
2. Tachograms were processed by the HRV analysis module of VivoSense 1.0 (Vivonoetics, 2011), which can process multiple files in an automated "batch process" format. See Supplemental Digital Content 1 for details of processing and derivation of HRV measures.
3. Following the batch process, trained scorers (VR, AM) visually inspected each file in accordance with the Task Force's recommendations that automated HRV analyses should be followed by visual inspection and manual correction. Additional corrections were required in approximately 8%-10% of files and each corrected file was re-processed to generate HRV variables.
4. Files for which the software determined that there was insufficient artifact-free data to accurately calculate frequency-domain variables were excluded from further HRV analysis. Typically this occurred when there was prominent motion artifact throughout the 5-minute session. Table 1 displays the percentages of sufficient artifact-free HRV data in each cohort.

HRV measures were generated per the recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (14) and included [definitions and physiological correlates can be found in (3, 14) and specified citations]:

1. HR: Heart rate in beats/minute over the 5-minute session; a time-domain measure;
2. SDNN: standard deviation of the R-R intervals in ms; a time-domain measure influenced by both sympathetic and parasympathetic activity;
3. RMSSD: root mean square successive differences between R-R intervals; a time-domain measure primarily influenced by parasympathetic activity;
4. VLF: absolute power of the very low frequency (< 0.04 Hz) band in ms^2; a frequency-domain measure whose physiological correlates are not well-understood;
5. LF: absolute power of the low frequency (0.04-0.15 Hz) band in ms^2; a frequency-domain measure thought to reflect sympathetic activity and some parasympathetic activity (34, 35);
6. HF: absolute power of the high frequency (0.15-0.4 Hz) band in ms^2; a frequency-domain measure thought to reflect primarily parasympathetic activity (34, 35);
7. LFnorm: LF power in normalized units calculated by LF/(total power-VLF); a frequency-domain measure. LFnorm reflects the percentage of total power that is accounted for by LF which reflects both sympathetic and parasympathetic activity;
8. HFnorm: HF power in normalized units calculated by HF/(total power-VLF); a frequency-domain measure. HFnorm is thought to reflect percentage of total power that is accounted for by HF which reflects primarily parasympathetic activity;

9. LF/HF ratio: ratio of LF over HF; a frequency-domain measure. Higher ratios have been proposed to reflect more sympathetic relative to parasympathetic activity (36). It is important to note, however, that use of the LF/HF ratio as a robust measure of sympathetic to parasympathetic balance has come under substantial scrutiny. Eckberg provides a review of the evidence that parasympathetic contributions to LF are significant and that HF may not reflect parasympathetic function when respiration is not controlled (4).

Of these nine variables, the LF/HF ratio and the transformed values for SDNN, RMSSD, LF, and HF were used in further analysis. HR was not used because, in relation to the other measures, is not a direct index of HRV. The LF/HF ratio is a widely used index that is the ratio of LFnorm and HFnorm, thus these latter variables were not further analyzed. Guidelines on the selection of appropriate epochs for assessing HRV suggest that a 5-minute recording window is sufficient to derive LF and HF as well as time-domain indices such as SDNN and RMSSD, but accurate assessment of VLF likely requires at least a 50-minute recording window (3, 14). Thus, VLF was not included in subsequent analyses.

Variables studied in relationship to HRV were as follows: age in years, ancestry, hours since last nicotine use (for participants who reported using nicotine within 24 hours), hours since last caffeine use (for participants who reported using caffeine within 24 hours), body mass index (BMI) in kg/m^2, history of traumatic brain injury (TBI) as defined by a self-report of head injury that was accompanied by either a loss of consciousness or altered mental status, and current use of psychotropic medications (Table 2). This latter category was defined broadly as the current use of one or more of the following classes of medications: antidepressants, benzodiazepines, sleep aids, mood stabilizers, prescription stimulants, or antipsychotic medications. Beck Depression Inventory-II (BDI) (37) scores and the Clinician-Administered PTSD Scale (CAPS) (38) scores were also assessed in relation to HRV. The CAPS is a clinician-administered structured interview and is considered the "gold standard" for assessment of PTSD symptoms and ascertainment of a PTSD diagnosis using DSM-IV criteria (see below).

Ancestry was determined using genetic information as described in (39). In brief, genotypes of 1,783 ancestry-informative markers (AIMs) were used to determine a participant's ancestry at the continental level for the 7 geographic regions Africa, Middle East, Europe, Central/South Asia, East Asia, Americas, and Oceania. Ancestry estimates were determined using STRUCTURE v2.3.2.1. (40) at K=7, including prior population information of the HGDP reference set (41). Based on these ancestry estimates, MRS participants with HRV data were placed into 4 groups: participants with >95% European ancestry were grouped with European-Americans (N=1503); participants with >5% African ancestry and <5% each Native American, Central Asian, East Asian, and Oceanic ancestry as African-American (N=145), participants with >5% Native American and <10% African, and <5% each Central Asian, East Asian and Oceanic ancestry as Hispanic and Native Americans (N=434), and all others, including 43 East Asians with >95% East Asian ancestry as Others (N=348).

Statistical Analysis

Frequency and descriptive statistics were generated for each HRV measure (Table 3). In cases where a variable showed significant skew, transformations were applied per the recommendations of Tabachnick and Fidel (42). Natural log transformations of frequency-domain measures such as LF, HF, and the LF/HF ratio are widely used (11, 13, 21, 43-45). Following transformation of the appropriate variables, outliers defined as greater than 3 standard deviations from the mean were excluded from subsequent data analyses (Table 3). SDNN and RMSSD showed a moderate positive skew and square root transformations of these variables were generated (42). LF, HF, and the LF/HF ratio showed a substantial positive skew and natural log transformations were generated. After the log transformations, distributions were inspected and skew was assessed. The log-transformed HRV values were normally distributed. Distributions of HRV were relatively similar across the four cohorts (though differences in mean HRV measures were observed between cohorts; see Table 4 and below).

A high proportion of Marines had BDI and CAPS scores of zero (22.3% for BDI, 15.7% for CAPS); hence categorical variables were created for BDI and CAPS scores. For the BDI, three categories were generated: BDI scores of 0, BDI scores between 1 and 19 suggesting minimal/mild depression, and BDI scores of 20 or greater suggesting moderate/severe depression. For the CAPS, Marines were categorized as having no symptoms of PTSD, meeting partial criteria for PTSD, or meeting full criteria for PTSD. Full criteria are derived from the DSM-IV diagnosis for PTSD and require the following: at least 1 "B" symptom (the traumatic event is persistently re-experienced), 3 "C" symptoms (persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness), and 2 "D" symptoms (persistent symptoms of increased arousal). Participants were deemed to have met partial criteria for PTSD if they did not meet full criteria but did endorse: 1) at least 1 "B" symptom, 2 "C" symptoms, *and* 2 "D" symptoms; or endorsed 2) at least 1 "B" symptom, 3 "C" symptoms, *or* 2 "D" symptoms.

In order to assess the relationship between HRV and PTSD after accounting for important covariates, a single multinomial logistic regression was conducted with PTSD category (comparing no PTSD to partial PTSD and full PTSD) as the outcome variable and HRV and TBI history as predictors as well as age, cohort, and ancestry, because these variables were shown to have significant and consistent associations with HRV (see Table 4). A regression approach was chosen in order to account for multiple covariates while preserving power, and PTSD category was chosen as the outcome with the hope that this approach could most sensitively detect potential HRV changes associated with different severity levels of PTSD. Cases with no PTSD were used as the reference category. As above, a large proportion of zero CAPS scores precluded the use of CAPS scores as a continuous measure of PTSD severity. In order to minimize multicolinearity among the highly intercorrelated HRV variables, one HRV index, log-transformed HF, was entered as a predictor. The assumptions of the model were tested with goodness-of-fit Pearson and Deviance Chi-Squares. Significance values were both $p > .100$, indicating that the data were consistent with model assumptions.

To assess whether there was a PTSD-by-depression interaction on HRV, a two-way analysis of variance (ANOVA) was conducted with PTSD category (no PTSD versus full or partial PTSD) and BDI category (no or minimal/mild depression vs. moderate/severe depression) as the independent variables and log-transformed HF as the dependent variable.

To provide simple descriptive statistics across diagnostic groups, HRV differences with respect to presence or absence of a DSM-IV diagnosis of PTSD as defined by the CAPS were assessed using an independent samples t-test, and HRV differences between the three BDI categories (no depression, minimal/mild, and moderate/severe) were assessed using a Univariate ANOVA.

Relationships between HRV and symptoms scores as well as the continuous variables of age, BMI, hours since nicotine use, hours since caffeine use, and scores on the Alcohol Use Disorders Identification Test (AUDIT) (46) were assessed with Pearson R correlation coefficients. HRV differences with respect to presence or absence of a TBI, history and presence or absence of psychotropic medication usage were analyzed using Independent sample t-tests. HRV differences between cohorts and ancestry categories were analyzed using Analyses of Variance (ANOVA). HRV differences between those with a history of a prior deployment and those without a deployment history were analyzed using Analysis of Covariance (ANCOVA) with age as a covariate and PTSD group as a factor. It should be noted that cohort (1-4) had significant associations with both HRV variables and outcome measures of interest (BDI, CAPS); hence our descriptive statistics are presented by cohort. The four cohorts were tested during four different periods and uncontrolled factors such as season, differences in training, and differences in specific deployment destination may have occurred. To account for this potential variance, cohort was consistently kept as a factor in our statistical models.

Significance levels were set at $p < 0.050$ and Cohen's d and partial Eta squared (η_p^2) effect sizes were calculated when relevant. Statistical analyses were conducted with PASW/SPSS 18.

Results

Description of HRV

The range of the HRV measures (Table 3) were generally consistent with HRV values reported in a recent review of 44 short-recording HRV studies (47), with the exception of values of VLF, LF and HF, which are substantially higher in the current population than what has been previously reported.

Relationship of HRV to depression and PTSD

Across all four cohorts, 1678 (69%) participants were categorized as having minimal to mild depression while 189 (7.8%) participants were categorized as having moderate to severe depression. The ANOVA yielded no significant differences or trends among the three BDI categories on any of the HRV measures.

Across all four cohorts, 120 Marines (4.9%) were categorized as meeting the full criteria for PTSD while 195 Marines (8.0%) were categorized as meeting partial PTSD criteria. The multinomial logistic regression with PTSD category as the outcome and HRV, TBI group and other covariates as predictors was statistically significant (Chi-Square = 77.7, $p < .001$, pseudo-R^2=.03). HRV reached statistical significance in its association with partial PTSD and full PTSD (Table 5) such that log-transformed HF values were lower in individuals with partial and full PTSD compared to individuals without PTSD. As a confirmatory post-hoc analysis, an ordinal regression was conducted with PTSD category (no PTSD, partial PTSD, full PTSD) as the ordinal outcome. The proportional odds assumption was met (Test of Parallel lines Chi-Square = 11.8, $p < .100$). The model was statistically significant (Chi-Square = 65.5, $p < .001$), and again HF was statistically significant in its association with PTSD (ordered log-odds estimate = -.18, $p = .004$) when other covariates were also in the model.

The two-way ANOVA with PTSD group and depression group as independent variables and log-transformed HF as the dependent variable indicated no significant PTSD-by-depression interaction [$F(1,2387)=.18$, ns, $\eta_p^2<.001$)] nor a main effect of depression group [$F(1,2397)=.05$, ns, $\eta_p^2 < .001$). Figure 1A displays back-transformed HF means comparing subjects with no PTSD and no depression, subjects with depression but no PTSD, subjects with full or partial PTSD and depression, and subjects with full or partial PTSD but no depression. The main effect of PTSD group did not reach statistical significance [$F(1,2397)=2.55$, $p=.111$, $\eta_p^2 = .001$), but post-hoc comparisons of the four groups indicated that the PTSD-only group had significantly lower HF than the group without PTSD and depression ($p = .012$).

Follow-up Pearson correlations within full and partial PTSD cases indicated that HF was not significantly associated with any specific symptom CAPS symptom domains (re-experiencing $r = -.02$, ns, avoidance $r = -.001$, ns, or arousal $r= .02$, ns). A Pearson correlation also indicated that total CAPS scores were significantly positively correlated with BDI scores ($r= .54$, $p < .001$), even when subjects with BDI and CAPS scores of zero were excluded from the correlation ($n= 1655$, $r = .49$, $p < .001$).

The 120 participants across all four cohorts who met full diagnostic criteria for PTSD were compared to participants without full or partial PTSD (n=2115) on the selected HRV measures using independent samples t-tests. Levene's tests for Equality of Variances were non-significant for all five HRV measures. Marines with PTSD had significantly lower RMSSD [$t(2210)=2.2$, $p = .027$, $d = .21$], lower LF [$t(2207)=2.6$, $p = .010$, $d = .24$], and lower HF [$t(2213)=2.5$, $p = .013$, $d = .23$] than study participants without full or partial PTSD. The LF/HF ratio did not significantly differ among the two groups [$t(2217)=.82$, ns, $d = .08$], nor did SDNN [$t(2218)=1.7$, $p = .087$, $d = .16$).

Relationship of HRV variables to covariates

The relationship between at least one of the selected HRV measures and the following variables reached statistical significance: cohort, age, ancestry, hours since nicotine use, BMI, history of TBI, psychotropic medication use, and history of prior deployments (Table 4). Because cohort had significant associations with many HRV variables, we consistently

kept it in as a factor in our subsequent regression models. Variables associated with lower HRV included older age, non-Caucasian ancestry, BMI, recency of nicotine use, and TBI history. Figure 1B displays back-transformed HF means of no PTSD versus partial or full PTSD subjects with and without a TBI history. There were small effect sizes for HRV differences between participants who were taking psychotropic medications and those who were not such that psychotropic medication users had, on average, higher HRV. Finally, when age was used as a covariate, Marines with a history of prior deployment had significantly lower HF than those without a deployment history. Although the deployment history-by-PTSD-group interaction was not statistically significant [$F(2,2402=.72$, ns], planned comparisons indicated that HF was significantly lower in previously deployed participants without PTSD versus participants without PTSD who did not have a previous deployment ($p= .033$). This difference did not reach statistical significance in those with partial PTSD or full PTSD (Figure 2). It is detailed in Figure 2 that the sample sizes for the comparisons in the partial and full PTSD groups were substantially smaller than those for the no PTSD comparisons. Figure 2 displays back-transformed HF means for Marines with and without a deployment history delineated by PTSD groups and adjusted for age.

Discussion

We have described a relatively non-obtrusive and rapid methodology to assess, process, and analyze HRV in a large population of military service members who, because of their eventual deployment to combat zones, are at risk of developing stress-related conditions. Furthermore, we have tested the relationship between HRV and PTSD while accounting for a history of traumatic brain injury and depression symptoms. TBI is highly prevalent in military service members of the recent conflicts in the Middle East, and the presence of TBI and PTSD together have been identified as the "signature injury" of these wars (48). The overlap between TBI and PTSD in military service members is further exemplified by the recent observation that deployment-related TBI is a strong predictor of deployment-related PTSD (49). Further, there are known effects of head injuries on HRV (albeit not strongly observed in our study, see below). Despite this knowledge, no study on PTSD and HRV in a military population has controlled for TBI history. The current results suggest that, even when a TBI history is accounted for, lower HRV is significantly associated with PTSD.

Depressive symptoms, however, were not related to HRV in this sample, and contrary to our hypothesis, the co-occurrence of PTSD and depressive symptoms was not associated with lower HRV than either condition alone even though PTSD symptom severity and depressive symptom severity were highly related. Rather, the group with PTSD in the absence of moderate or severe depressive symptoms had the lowest HRV. This finding is in contrast to what has been previously observed. Studies in non-military trauma survivors suggest that a trauma history and depression interact in their influence on autonomic arousal; for example the presence of depression with a trauma history was associated with lower respiratory sinus arrhythmia than either condition alone in females with a history of trauma related to crime, natural disaster, or assault (19). In a study of survivors of Hurricane Katrina, depression was more strongly associated with lower HRV than was PTSD (20), a finding in notable contrast to the current results. While depression was not related to lower HRV in the current study, a prior history of deployment was, even in Marines without a PTSD diagnosis, reminiscent of

a recent report of an association between combat exposure and decreased HRV (13). Thus, the relationships among depressive symptoms, trauma symptoms, and autonomic nervous system function may vary depending on the population of trauma survivors and is highly dependent on the nature and context of the traumatic event.

Several factors could explain the absence of a relationship between depressive symptoms and HRV, one of which is the inherent limitation of a self-report instrument. Furthermore, our classification of BDI scores into just three categories, while necessary to achieve adequate sizes of groups, may have been too coarse to detect more subtle HRV differences. Lastly but importantly, the BDI is sensitive in capturing the severity of acute depressive symptoms but is less informative about the chronicity of depression. Most findings relating depression to HRV have been conducted on individuals with a chronic depression condition (7, 19). Nevertheless, the current findings suggest that there remains a high co-occurrence of symptoms of depression with post-traumatic symptoms, which is an immediate public health concern in active-duty military personnel. The causal directions of these relationships have not been well-elucidated.

Marines with a history of TBI with associated altered mental status or loss of consciousness demonstrated lower HRV, but the effect size for this finding was very small. The proportion of Marines reporting a previous TBI was relatively high (55.5%) but was consistent with the published characteristics of this cohort of Marines (27). Many of these cases are likely "mild" head injuries given that either altered mental status or loss of consciousness was sufficient to identify a TBI. It is also important to note that participants were asked about a history of any head injury, not solely those related to prior military deployments, which likely accounted for the high percentage of TBIs in this sample as compared to other studies (17). Head injury has been associated with alterations in the regulation of the cardiac system and lower HRV (18, 50, 51). Interestingly and potentially relevant to the current findings, HRV abnormalities in athletes with a recent concussion were only observed during an exercise session, but not at rest (52). The severity of a TBI as well as time elapsed since the injury are also likely to be important factors in the normalization of HRV (51).

Other factors that were associated with HRV and are relevant to consider in future analyses with these cohorts included age, ancestry, body mass index, and nicotine use. The relationship between age and HRV has been widely observed. The genetic determination of ancestry underscores previous observations that genetic factors are thought to contribute to a substantial proportion of the variance (13-23%) in HRV, albeit less so than the combined influence of non-genetic variables such as age, sex, and environmental factors (53). BMI was only weakly related to one HRV measure, the high-frequency index. BMI is known to be a relatively less reliable index of fat accumulation in athletes with high muscle mass (54). Active-duty Marines readying for deployment to a combat zone fall into this category. The high fitness levels and relative youth of these participants may also account for the higher values of HRV observed in this study compared to other published reports. Recent use of nicotine was related to lower HRV, supporting previous work that nicotine use may alter autonomic functioning (24).

Mechanisms underlying lower HRV in PTSD has been postulated to reflect reduced vagal or parasympathetic tone (14). Diminished parasympathetic tone may accompany changes in amygdala and medial prefrontal cortex activation, brain regions that have been implicated in PTSD and are thought to underlie fear and threat responses (55, 56). Changes in LF and HF have been associated with altered connectivity between the aforementioned brain regions and structures such as the anterior cingulate and insula that are implicated in orienting attention and vigilance (56, 57). In any interpretation of findings related to LF, HF, and the LF/HF ratio, however, it is important to consider the following caveat. There has traditionally been an overreliance on these indices as being direct reflections of sympathetic/vagal balance. As Eckberg (4) reviews, parasympathetic contributions to LF are significant, changes in HF may not always be explained by changes in parasympathetic activity, and under certain conditions sympathetic and parasympathetic changes occur in parallel to one another and not reciprocally. Thus caution should be used in making firm conclusions about the physiological underpinnings of sympathetic versus parasympathetic functioning in the absence of rigorous experimental controls.

Other limitations of this study include a restricted age range and a lack of female participants, limiting the generalizability of our findings to a relatively young, athletic group of males. This sample is, we would argue, highly representative of US service members currently at greatest risk for combat-related PTSD. Another limitation is that there were small but significant differences ($\eta_p^2 = .003$-$.054$) between cohorts in HRV and other demographic variables that may have been due to chance or may have been attributable to a number of random factors such as differences in season of testing, individual battalion demographics, and physical training courses leading up to or prior to the data collection. The relationship between HRV measures and PTSD caseness, however, was robust enough to be significant even when controlling for cohort. Several potential experimental factors which can affect HRV are relevant to mention. First, participants were asked to attend to video images of hidden pictures. Previous studies have reported cardiac deceleration during reaction time and response inhibition tests [see (58) for a summary]. Whereas participants in this study were told that they would not be required to respond to the visual images, the paradigm could arguably represent a cognitive challenge that affected HRV. We cannot entirely rule out that cognitive activity may have affected HRV, which would be a consistent phenomenon across all participants in the study. Second, no measure of respiration was obtained during HRV assessment, which is a notable limitation since changes in breathing rates are directly related to respiratory sinus arrhythmia (3) and can also be associated with different mood states, e.g., higher respiratory rates in anxious patients (59). The collection of large sample sizes of Marines in short time frames rendered PPG a practical rapid method as opposed to use of ECG Holter monitors plus respiratory band application and recordings. Thus we were not able to assess to what extent breathing rates in participants with PTSD may have moderated HRV. A final limitation is that this study is cross-sectional, however prospective analysis of these cohorts upon return from deployment and after onset of PTSD symptoms is ongoing.

In conclusion, we have described our methodology for the collection and analysis of short-term HRV in a large population of Marines readying for deployment. Lower HRV was observed in participants with a full or partial PTSD diagnoses at the pre-deployment MRS

time frame even when TBI history was accounted for. Previous deployments were associated with lower HRV while depression was not, but depression was more strongly related to PTSD than it was to HRV. Future longitudinal analyses of these military service members will include the consideration of the HRV factors and covariates elucidated here. The ultimate aim of this research is to uncover whether ANS functions can predict who is vulnerable and who is resilient, or whether ANS functions emerge in tandem with mental health effects of combat exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIMS	ancestry-informative markers
ANS	autonomic nervous system
AUDIT	Alcohol Use Disorders Identification Test
BDI	Beck Depression Inventory
BMI	body mass index
CAPS	Clinician-Administered PTSD scale
EMG	electromyography
HF	high frequency index
HR	heart rate
HRV	heart rate variability
LF	low frequency index
MRS	Marine Resiliency Study
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
OND	Operation New Dawn

PPG	photoplethysmograph
PTSD	posttraumatic stress disorder
SA	sinoatrial node
SSRI	selective serotonin reuptake inhibitor
TBI	traumatic brain injury
VLF	very low frequency index

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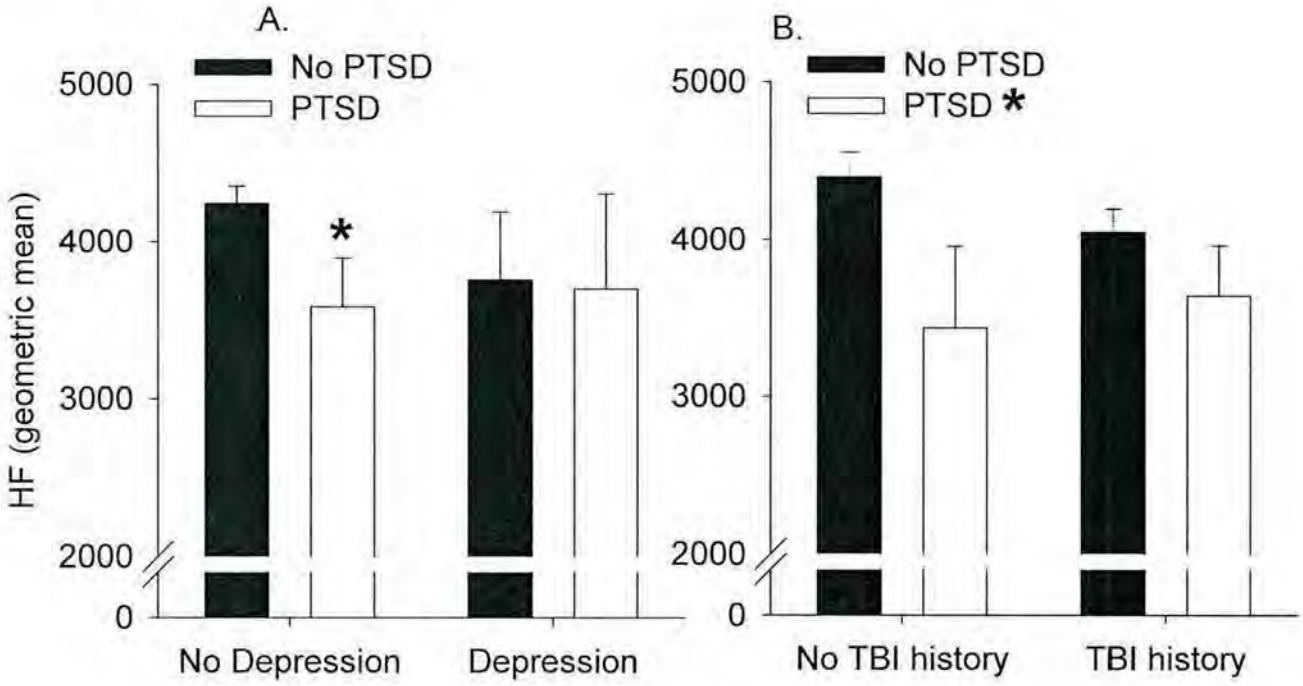

Figure 1.

Mean high frequency index (HF) in Marines with and without PTSD and depression or TBI, all cohorts combined.

Note: Error bars are standard errors of the mean. Values in figure are back-transformed absolute HF means. Left Panel (A): * $p < .050$, PTSD only versus no PTSD/no depression in post-hoc comparison with transformed HF values as the dependent variable. Sample sizes are as follows: no PTSD and no depression n = 1965, PTSD only n = 248, depression only n = 124. PTSD and depression n = 64. "PTSD" refers to partial or full PTSD cases as determined by the CAPS. "Depression" refers to moderate/severe depression as determined by the BDI. Right Panel (B): * $p < .050$, main effect of PTSD versus no PTSD in ANOVA with transformed HF values as the dependent variable. Sample sizes are as follows: no PTSD and no TBI history n = 981, PTSD and no TBI history = n = 87, no PTSD and TBI history, n=1113, PTSD and TBI history n= 228. "PTSD" refers to partial or full PTSD cases as determined by the CAPS. History of TBI= self-reported history of a head injury accompanied by either loss of consciousness or altered mental status.

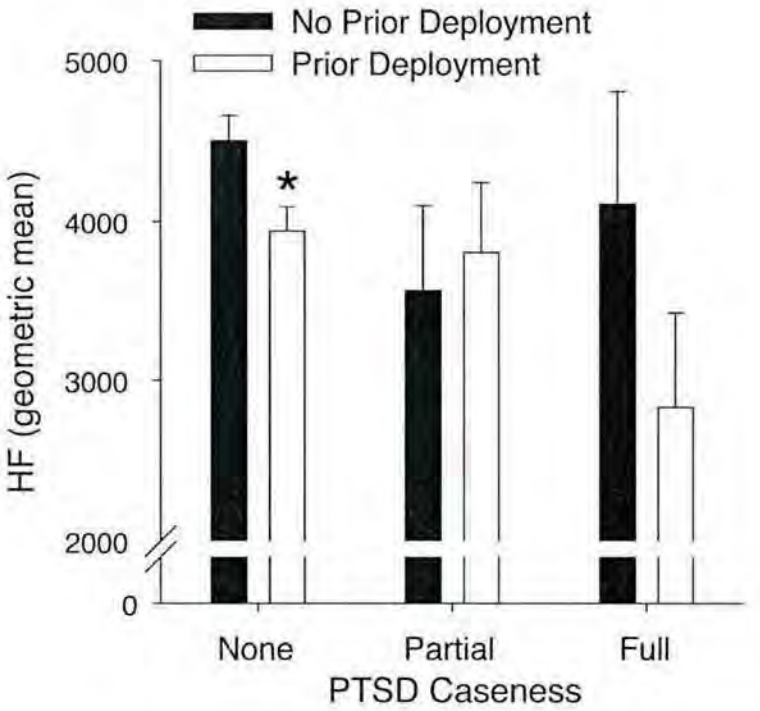

Figure 2.

Mean high frequency index (HF) for no history of prior deployments versus prior deployment history in Marines with no PTSD, partial PTSD, and full PTSD, all cohorts combined.

Note: Errors bars are standard errors of the mean. Values in figure are back-transformed absolute HF means. *$p < .050$ vs. No PTSD/No Prior Deployment group using ANCOVA with transformed HF values as the dependent variable and age as a covariate. Sample sizes are as follows: no PTSD and no prior deployment n = 1031, no PTSD and prior deployment n = 1063, partial PTSD and no prior deployment n = 80, partial PTSD and prior deployment n = 116, full PTSD and no prior deployment n = 51, full PTSD and prior deployment n = 68. See results for details.

Table 1

Cohort sizes for heart rate variability (HRV) data collected in active duty Marines before deployment to OIF/OEF/OND.

	N eligible participants in cohort	N (%) participants with sufficient artifact-free HRV data
Cohort 1	315	298 (94.6%)
Cohort 2	721	699 (96.9%)
Cohort 3	670	603 (90.1%)
Cohort 4	886	830 (93.7%)
Total	2592	2430 (93.8%)

Table 2

Demographic and descriptive information for participants in each cohort.

	Cohort 1 (N=298)	Cohort 2 (N=699)	Cohort 3 (N=603)	Cohort 4 (N=830)	Total (N=2430)
Age (years)	21.9 (3.1)	22.4 (3.4)	23.2 (3.7)	23.1 (3.7)	22.8 (3.5)
Ancestry	62.8% European-American 3.7% African-American 19.1% Native-American/ Mexican 14.4% Asian/Other	66.5% European-American 4.7% African-American 15.5% Native-American/ Mexican 13.2% Asian/Other	60.7% European-American 6.5% African-American 17.2% Native-American/ Mexican 15.6% Asian/Other	58.4% European-American 7.5% African-American 19.8% Native-American/ Mexican 14.3% Asian/Other	61.9% European-American 6.0% African-American 17.9% Native-American/ Mexican 14.3%Asian/Other
Body Mass Index (kg/m^2)	26.8 (3.4)	27.3 (3.0)	28.1 (3.4)	27.8 (3.3)	27.6 (3.2)
Number (%) with a history of TBI	n=188 (63.1%)	n=422 (60.3%)	n=326 (54.1%)	n=413 (49.8%)	n=1349 (55.5%)
Hours since nicotine use[a]	n=133 4.0 (4.5)	n = 371 3.3 (4.2)	n = 284 2.7 (3.1)	n = 390 2.6 (3.3)	n = 1178 3.0 (3.7)
Hours since caffeine use[a]	n = 189 7.6 (6.7)	n = 460 6.6 (6.3)	n = 293 6.1 (2.7)	n = 419 5.0 (5.6)	n = 1361 6.1 (6.1)
AUDIT (alcohol use) total score	8.2 (6.3)	8.4 (7.4)	7.6 (6.7)	6.9 (6.3)	7.7 (6.8)
Number (%) using psychotropic medications	n=7 (2.3%)	n=12 (1.7%)	n=7 (1.2%)	n=4 (0.5%)	n= 30 (1.2%)
BDI (depression) total score	7.5 (8.3)	8.5 (8.8)	6.2 (7.3)	5.2 (6.5)	6.7 (7.8)
CAPS (PTSD) total score	16.9 (16.8)	16.8 (15.6)	14.6 (15.8)	13.2 (14.6)	15.0 (15.5)
Number (%) with PTSD	n=14 (4.7%)	n=41 (5.9%)	n=36 (6.0%)	n=29 (3.5%)	n=120 (5.0%)
Number (%) with a prior deployment	n=161 (54%)	n=386 (55.2%)	n=281 (46.5%)	n=426 (51.3%)	n=1254 (51.7%)

Note: History of TBI= self-reported history of a head injury accompanied by either loss of consciousness or altered mental status. AUDIT= Alcohol Use Disorders Identification Test. BDI= Beck Depression Inventory, CAPS=Clinician-Administered PTSD Scale. PTSD diagnosis was determined by meeting full DSM-IV criteria as assessed by the CAPS. Values in the table are means and (standard deviations) unless specified otherwise.

[a] calculated only in subjects who self-reported use of this substance within 24 hours.

Table 3

Descriptive information for heart rate variability variables in the entire sample (N=2430).

	Heart Rate	SDNN	RMSSD	VLF	LF	HF	LFnorm	HFnorm	LF/HF Ratio
Mean (SD)	67.1 (10.2)	64.1 (26.9)	58.9 (34.5)	2380.2 (2470.9)	5144.1 (5467.4)	4153.8 (5074.4)	.56 (.17)	.41 (.16)	1.9 (1.7)

Note: Heart rate is in beats/min. SDNN =standard deviation of the R-R intervals in ms. RMSSD =root mean square successive differences between R-R intervals. VLF= absolute power of the very low frequency (< 0.04 Hz) band in ms^2. LF = absolute power of the low frequency (0.04-0.15 Hz) band in ms^2. HF = absolute power of the high frequency (0.15-0.4 Hz) band in ms^2. LFnorm= LF power in normalized units calculated by LF/(total power-VLF). HFnorm= HF power in normalized units calculated by HF/(total power-VLF). LF/HF ratio= ratio of LF over HF. For a given variable in a given cohort, a range of 0-9 subjects were moved due to outlier scores, for an average 0.34% removed due to outlier scores.

Table 4

Relationship of heart rate variability variables to demographic and other factors.

	SDNN	RMSSD	LF	HF	LF/HF ratio
Cohort	$F(3,2419)=45.68^{***}$, $\eta_p^2 = .054$ Cohort 1>Cohort 3,4 > Cohort 2	$F(3,2411)=34.65^{***}$, $\eta_p^2 = .041$, Cohort 1>Cohort 3,4 > Cohort 2	$F(3,2408)=25.59^{***}$, $\eta_p^2 = .031$ Cohort 1>Cohort 3,4 > Cohort 2	$F(3,2414)=15.56^{***}$, $\eta_p^2 = .019$ Cohort 1>Cohort 3,4 > Cohort 2	$F(3,2419)=2.06$, $\eta_p^2 = .003$ Cohort 1>Cohort 3[b].
Age (years)	Pearson $r = -.11^{***}$	Pearson $r = -.12^{***}$	Pearson $r = -.11^{***}$	Pearson $r = -.16^{***}$	Pearson $r = .09^{***}$
Ancestry	$F(3,2419)=2.54^{*}$, $\eta_p^2 = .003$ Caucasian> African-American, Asian/Other	$F(3,2411)=.37$, $\eta_p^2 = .000$	$F(3,2408)=8.48^{***}$, $\eta_p^2 = .010$ Caucasian>African-American, Native-American/ Mexican, Asian/Other	$F(3,2414)=1.74$, $\eta_p^2 = .002$ Caucasian>Asian/Other[b].	$F(3,2419)=7.10^{***}$, $\eta_p^2 = .009$ Caucasian>African-American, Native-American/Mexican, Asian/Other
Body Mass Index (kg/m²)	Pearson $r = -.04$	Pearson $r = .01$	Pearson $r = -.07^{**}$	Pearson $r = -.01$	Pearson $r = -.08^{***}$
History of TBI	$t(2413)=.03$, $d = .001$	$t(2405)=.44$, $d = .02$	$t(2402)=.17$, $d=.01$	$t(2408)=1.91$, $d = .08$	$t(2413)=2.44^{*}$, $d = .10$ TBI history> no TBI
Hours since nicotine use[a]	Pearson $r = .10^{***}$	Pearson $r = .09^{**}$	Pearson $r = .09^{**}$	Pearson $r = .07^{*}$	Pearson $r = -.01$
Hours since caffeine use[a]	Pearson $r = .05$	Pearson $r = .03$	Pearson $r = .02$	Pearson $r = .02$	Pearson $r = .01$
AUDIT total score	Pearson $r = .01$	Pearson $r = .02$	Pearson $r = -.01$	Pearson $r = .01$	Pearson $r = -.01$
Use of psychotropic medications	$t(2422)=1.50$, $d=.28$ med users> non-users	$t(2412)=1.28$, $d=.24$ med users>non-users	$t(2409)=2.03^{*}$, $d=.38$ med users> non-users	$t(2415)=1.47$, $d=.27$ med users> non-users	$t(2420)=.17$, $d=.03$
History of a prior deployment[c]	$F(1,2407)=2.52$, $\eta_p^2 = .001$	$F(1,2399)=2.95$, $\eta_p^2 = .001$	$F(1,2396)=2.02$, $\eta_p^2 = .001$	$F(1,2402)=5.13^{*}$, $\eta_p^2 = .002$, prior < no prior	$F(1,2407)=0.45$, $\eta_p^2 < .001$

Note: SDNN and RMSSD were square root-transformed. VLF, LF, HF, and the LF/HF ratio were natural log-transformed. Direction of findings is reported if statistical significance is reached in either the overall test or planned comparisons, or at least a small effect size is achieved. History of TBI= self-reported history of a head injury accompanied by either loss of consciousness or altered mental status. AUDIT= Alcohol Use Disorders Identification Test. SDNN =standard deviation of the R-R intervals in ms. RMSSD =root mean square successive differences between R-R intervals. LF = absolute power of the low frequency (0.04-0.15 Hz) band in ms^2. HF = absolute power of the high frequency (0.15-0.4 Hz) band in ms^2. LF/HF ratio= ratio of LF over HF.

η_p^2 = partial Eta squared. d= Cohen's d effect size.

[a] calculated only in subjects who self-reported use of this substance within 24 hours.

[b] overall ANOVA did not achieve statistical significance but this planned comparison was significant at $p < .05$.

[c] ANCOVAs for history of prior deployment were conducted with age as a covariate and PTSD group (no PTSD vs. partial PTSD vs. full PTSD) as a factor.

*** $p < .001$

** $p < .010$

* $p < .050$

Table 5

Parameter estimates for the high frequency index (HF) in the multinomial logistic regression predicting no PTSD diagnosis (n=2115) versus partial PTSD diagnosis (n= 195) versus full PTSD diagnosis (n=120).

PTSD group	B	Std. Error	Wald χ^2	df	Significance	Exp (B) Odds Ratio	95% CI lower for Exp (B)	95% CI upper for Exp (B)
Partial PTSD	−.16	.08	4.44	1	0.035	.85	.94	1.02
Full PTSD	−.20	.09	4.44	1	0.035	.82	.67	.99

Note: The following covariates were first entered into the model: age, ancestry, cohort, and history of TBI. HF values were log-transformed. PTSD groups were determined by the CAPS. Partial PTSD= did not meet full criteria and had one of two CAPS profiles: 1) at least 1 "B" symptom, 2 "C" symptoms, *and* 2 "D" symptoms; or 2) at least 1 "B" symptom, 3 "C" symptoms, *or* 2 "D" symptoms. Full PTSD = at least 1 "B" symptom, 3 "C" symptoms, and 2 "D" symptoms. χ^2 = Wald Chi-Square

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The catecholamine biosynthetic enzyme dopamine β-hydroxylase (DBH): first genome-wide search positions trait-determining variants acting additively in the proximal promoter

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Dopamine beta-hydroxylase (DBH) is the biosynthetic enzyme catalyzing formation of norepinephrine. Changes in DBH expression or activity have been implicated in the pathogenesis of cardiovascular and neuropsychiatric disorders. Genetic determination of DBH enzymatic activity and its secretion are only incompletely understood. We began with a genome-wide association search for loci contributing to DBH activity in human plasma. Initially, in a population sample of European ancestry, we identified the proximal *DBH* promoter as a region harboring three common trait-determining variants (top hit rs1611115, $P = 7.2 \times 10^{-51}$). We confirmed their effects on transcription and showed that the three variants each acted additively on gene expression. Results were replicated in a population sample of Native American descent (top hit rs1611115, $P = 4.1 \times 10^{-15}$). Jointly, *DBH* variants accounted for 57% of DBH trait variation. We further identified a genome-wide significant SNP at the *LOC338797* locus on chromosome 12 as *trans*-quantitative trait locus (QTL) (rs4255618, $P = 4.62 \times 10^{-8}$). Conditional analyses on *DBH* identified a third genomic region contributing to DBH variation: a likely *cis*-QTL adjacent to *DBH* in *SARDH* (rs7040170, $P = 1.31 \times 10^{-14}$) on chromosome 9q. We conclude that three common SNPs in the *DBH* promoter act additively to control phenotypic variation in DBH levels, and that two additional novel loci (*SARDH* and *LOC338797*) may also contribute to the expression of this catecholamine biosynthetic trait. Identification of *DBH* variants with strong effects makes it possible to take advantage of Mendelian randomization approaches to test causal effects of this intermediate trait on disease.

INTRODUCTION

Dopamine β-hydroxylase (DBH) is the final enzyme in norepinephrine biosynthesis, catalyzing the oxidative hydroxylation of dopamine to norepinephrine in the noradrenergic nerve endings of the central and peripheral nervous systems (1). In the bloodstream, DBH enzymatic activity is abundant, emerging from both the sympathetic terminals and the adrenal medullary chromaffin cells (1). As a result of exocytosis, DBH is co-released with norepinephrine from synaptic vesicles into extracellular space and thus can be found in plasma and cerebrospinal fluid (CSF) (2,3). The enzymatic activity of plasma or CSF DBH corresponds to the level of DBH protein, with plasma and CSF DBH correlating highly in humans (4,5). As such, DBH is of high interest to both the neuropsychiatric and cardiovascular field. Changes in DBH activity and/or genetic variants in the *DBH* gene have been implicated in the pathophysiology of major depression (6), ADHD (7,8), Parkinson (9) and Alzheimer's disease (10,11) and PTSD (12,13), potentially through changes in central catecholamine levels, whereas altered sympathoadrenal activity is thought to be implicated in the pathogenesis of hypertension and cardiovascular disease (14,15).

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In family and twin studies plasma DBH (pDBH) activity is highly heritable, relatively stable over time in the same person, and only minimally susceptible to environmental factors such as physical stress or drugs (16). Furthermore DBH activity shows highly variable inter-individual differences which are likely the result of genetic factors (5,17), with heritability estimates accounting for ~80–90% of the variation.

Linkage analysis with non-DNA markers has identified a single quantitative trait locus (QTL) for DBH activity in a region on chromosome 9 (9q34) (18,19) and the *DBH* gene was later mapped to that region (20,21). Sequencing analyses by Zabetian *et al.* (22,23) further characterized the molecular structure of *DBH* and identified a SNP in the promoter region (rs1611115/C-970T/formerly C-1021T), which explained a large ~35–52% inter-individual variation in pDBH activity, while functional polymorphisms (A197T in exon 3, A304S in exon 5 and R535C in exon 11) in the gene did only show a modest putative effect for R535C in these studies (see review in 16). Extended sequencing in the promoter region identified six common SNPs in the proximal promoter and showed functional properties in *in vitro* and *in vivo* experiments for rs1611115 and rs1989787 (C-2073T). A newer linkage study in families confirmed *DBH* as a major contributor of pDBH activity, but also suggested two additional loci, one in close proximity to *DBH* and the second on chromosome 20p12 (24).

Analysis of DBH levels in clinical populations reported racial differences in pDBH activities, with Blacks having lower levels than Whites (25). Genetic studies on the *DBH* locus, initially performed in populations of European ancestry, have then been extended to include subjects of African and Asian descent and confirmed rs1611115 as the polymorphism with the strongest effect (22,26).

Here, we performed the first genome-wide association study (GWAS), with goals to: (1) replicate and extend previous findings on *DBH* locus variation and its effect on pDBH activity, (2) extend the search to identify additional, *trans*-QTLs for pDBH activity levels and (3) expand ancestry studies to include subjects of Native American descent and Hispanic ethnicity. In addition, we further examined functional properties of genetic markers in the *DBH* promoter region displaying peak-association with plasma DBH activity, in transfected chromaffin cells as well as *in vivo*. We show that *DBH* variants with strong effects may be used in a Mendelian randomization (MR) approach to test causal effects of this intermediate trait on disease, such as cardiovascular and neuropsychiatric symptoms and disorders.

RESULTS

Genome-wide association study in subjects of European ancestry

An initial GWAS for plasma DBH activity was performed with genotypes of 341 subjects of European ancestry (European Americans, EAs). The mean pDBH level in the 341 EAs was 11.44 IU/L [standard deviation (SD) = 6.95] (Supplementary Material, Fig. S1). Genotypes underwent rigorous quality control and included a final set of 7 871 575 markers obtained by genotyping and imputation. Linear regression under an additive genetic model, incorporating appropriate covariates, resulted in a low genomic control inflation factor of $\lambda_{GC} = 1.002$. A quantile–quantile (QQ) plot is shown in Supplementary Material, Figure S2A. A table with all GWAS results is available in the Supplementary Material, Table S1.

Our analyses identified the *DBH* locus as genome-wide significant with the top hit for a directly genotyped SNP rs1611115 at $P = 7.2 \times 10^{-51}$ (Fig. 1A and Table 1). A regional association plot of the *DBH* locus showed 34 genome-wide significant *DBH* SNPs within the same linkage disequilibrium (LD) block (Fig. 1B). Of these, one SNP was found in an exon (synonymous SNP exm793933, $P = 1.023 \times 10^{-27}$), 22 were intronic and 11 were located upstream of *DBH*, including 3 common SNPs within a 3 kb region of the promoter (rs1076150, rs1989787 and rs1611115, shown in detail in Table 1, top part). Two of these promoter SNPs (rs1989787 and rs1611115) were known to be functional (see 14 and 15) and the functionality of rs1076150 was investigated below. The proportion of variability explained (R^2)

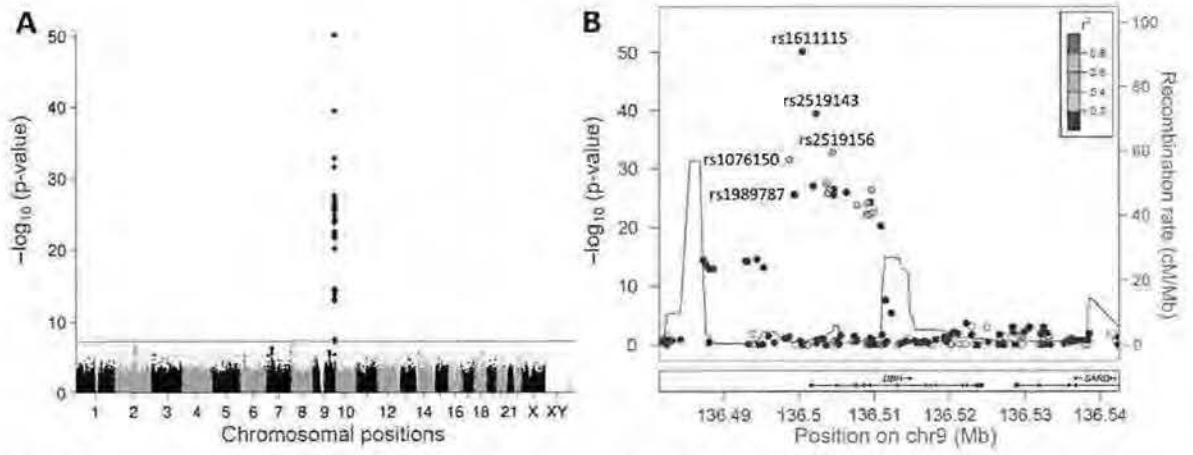

Figure 1. Results of the GWAS of plasma DBH activity in 341 subjects of European origin. (**A**) Manhattan plot showing the $-\log_{10}$ (*P*-values) for SNP associations with plasma DBH activity across the genome. The red horizontal line represents the genome-wide significance threshold at $P < 5 \times 10^{-8}$ and the dashed line represents suggestive evidence for association at $P < 5 \times 10^{-6}$. (**B**) Regional association plot, showing significant regions in *DBH* on chromosome 9. Directly genotyped SNPs are indicated by an asterisk (*). The SNPs are color coded based on the linkage disequilibrium with the most significant SNP rs1611115.

Table 1. Most significant hits in the genome-wide association study

Allele SNP	CHR	BP	Gene	Location	1/2[b]	EA GWAS Allele 1 freq.	Effect size	SE	*P*	NA descent GWAS Allele 1 freq.	Effect size	SE	*P*	Meta-analysis[a] *Q*	Effect size	*P*
rs1076150[c]	9	136498761	DBH	Upstream	T/C	0.512	−0.947	0.072	**2.74E−32**	0.710	−0.779	0.163	7.67E−06	0.35	−0.920	**1.38E−44**
rs1989787	9	136499412	DBH	Upstream	T/C	0.312	0.924	0.079	**2.13E−26**	0.196	0.747	0.191	1.92E−04	0.39	0.898	**1.50E−34**
rs1611115[c]	9	136500515	DBH	Upstream	T/C	0.248	−1.265	0.070	**7.20E−51**	0.317	−1.195	0.125	**4.10E−15**	0.63	**−1.248**	**4.60E−92**
rs7540659	1	100196119	FRRS1	Intron	T/A	0.341	−0.024	0.095	0.801	0.462	−0.793	0.160	**3.64E−06**	**0.00**	**−0.398**	0.301
rs60674788	2	35027196	CR617033	Downstream	C/G	0.257	−0.403	0.101	8.11E−05	0.354	−0.536	0.179	0.004	0.52	**−0.435**	**7.64E−07**
rs4459781	2	134204665	NCKAP5	Intron	C/T	0.281	−0.499	0.094	**2.15E−07**	0.215	−0.098	0.209	0.641	0.08	**−0.431**	**5.12E−07**
rs77518496	2	143629286	KYNU	Upstream	G/A	0.032	−0.012	0.232	0.959	0.115	1.252	0.253	**3.88E−06**	**0.00**	0.616	0.330
rs2351772	2	204079313	NBEAL1	Intron	C/T	0.418	0.346	0.091	1.71E−04	0.527	0.441	0.149	0.004	0.58	0.372	**1.61E−06**
rs112239800	2	232517876	BC069004	Downstream	G/A	0.102	0.509	0.149	7.38E−04	0.115	1.079	0.268	1.33E−04	0.06	0.645	**7.67E−07**
rs13095328	3	15226050	DIVA	Intron	C/T	0.100	−0.669	0.143	**3.97E−06**	0.059	0.634	0.354	0.077	**0.00**	**−0.058**	0.929
rs3774729[c]	3	63982082	ATXN7	Exon	A/G	0.323	0.000	0.086	0.996	0.290	0.869	0.164	**9.60E−07**	**0.00**	0.424	0.329
rs56030924	3	63995563	AK023371	Intron	A/G	0.286	0.017	0.090	0.848	0.269	0.885	0.164	**6.39E−07**	**0.00**	0.440	0.310
rs831692	3	64003983	PSMD6	Intron	A/G	0.310	0.031	0.089	0.731	0.288	0.893	0.166	**6.65E−07**	**0.00**	0.451	0.296
rs56237630	3	64049375	PRICKLE2	Downstream	A/C	0.145	−0.002	0.119	0.988	0.214	0.941	0.182	**1.52E−06**	**0.00**	0.460	0.329
rs12639432[c]	3	134770520	EPHB1	Intron	T/C	0.302	0.339	0.091	2.44E−04	0.462	0.546	0.150	4.71E−04	0.24	0.395	**4.16E−07**
rs7779937	7	10971712	NDUFA4	Downstream	A/G	0.048	−0.922	0.198	**4.74E−06**	0.016	0.643	0.660	0.333	**0.02**	**−0.266**	0.731
rs13242648	7	35777951	CR595224	Downstream	T/A	0.196	0.554	0.107	**3.92E−07**	0.136	−0.456	0.231	0.051	**0.00**	0.070	0.890
rs12701456	7	35827802	SEPT7	Upstream	C/T	0.196	0.547	0.107	**5.03E−07**	0.132	−0.389	0.231	0.096	**0.00**	0.101	0.828
rs13255006	8	1989315	MYOM2	Upstream	C/G	0.319	0.500	0.093	**1.52E−07**	0.172	−0.034	0.216	0.876	**0.02**	0.268	0.310
rs1338730	9	103520981	MURC	Downstream	C/T	0.402	−0.419	0.085	**1.15E−06**	0.253	−0.404	0.193	0.039	0.94	**−0.417**	**7.40E−08**
rs823919	9	104662606	GRIN3A	Upstream	A/G	0.124	0.582	0.127	6.56E−06	0.172	0.283	0.215	0.191	0.23	0.505	**3.98E−06**
rs7857468	9	136585380	SARDH	Intron	A/C	0.195	0.544	0.099	**8.09E−08**	0.170	0.142	0.236	0.549	0.12	0.484	**1.19E−07**
rs10795764	10	10238394	BC032914	Downstream	C/T	0.434	0.093	0.083	0.265	0.559	0.750	0.133	**2.33E−07**	**0.00**	0.413	0.208
rs870553	10	133970542	JAKMIP3	Intron	G/A	0.010	−1.210	0.417	0.004	0.059	−1.262	0.332	2.75E−04	0.92	**−1.242**	**1.77E−06**
rs112825992	10	134008571	DPYSL4	Intron	T/C	0.009	−1.252	0.450	0.006	0.055	−1.209	0.320	2.92E−04	0.94	**−1.223**	**2.65E−06**
rs4255618	12	131837477	LOC338797	Intron	C/A	0.353	0.388	0.088	1.26E−05	0.322	0.502	0.154	0.002	0.52	0.416	**4.62E−08**
rs8013529[c]	14	23649792	SLC7A8	Intron	G/A	0.139	−0.566	0.118	**2.37E−06**	0.059	0.320	0.328	0.332	**0.01**	**−0.176**	0.689
rs12595689	15	86009293	AKAP13	Intron	C/G	0.085	0.010	0.158	0.952	0.102	−1.198	0.222	**5.88E−07**	**0.00**	**−0.584**	0.333
rs117711052	17	74305308	QRICH2	Upstream	C/G	0.024	1.155	0.278	4.12E−05	0.016	1.567	0.627	0.014	0.55	1.223	**1.48E−06**
rs115172145	17	74310984	PRPSAP1	Intron	C/T	0.024	1.144	0.278	4.77E−05	0.016	1.567	0.627	0.014	0.54	1.213	**1.74E−06**
rs7228140	18	45907244	ZBTB7C	Intron	C/T	0.046	−0.941	0.194	**1.88E−06**	0.071	0.439	0.313	0.165	**0.00**	**−0.273**	0.692
Conditional analysis[d]																
rs7857468	9	136585380	SARDH	Intron	A/C	0.195	0.500	0.065	**2.38E−13**	0.170	0.489	0.155	0.002	0.946	0.498	**1.15E−16**
rs7040170[c]	9	136586367	SARDH	Intron	G/A	0.221	0.439	0.062	**7.82E−12**	0.177	0.456	0.153	0.004	0.918	0.442	**1.31E−14**

[a]Random-effects models were used for SNPs with significant heterogeneity *Q* values (bold), otherwise fixed-effects models.
[b]Allele 1 is the coding allele.
[c]Directly genotyped SNP.
[d]Regression analyses including *DBH* SNPs rs1076150, rs1989787 and rs1611115 as covariates.
P-values in bold meet suggestive ($P < 5.0E-06$) or genome-wide significance ($P < 5.0E-08$).

by the *DBH* gene, based on five highly significant *DBH* SNPs in low LD with each other plus the three (putative) functional promoter SNPs, was 0.569.

No other chromosomal region reached genome-wide significance. However, there were 10 regions which showed suggestive evidence ($P < 5 \times 10^{-6}$) in EAs. For each of these, the SNP with the lowest *P*-value is presented in Table 1 (middle part) and Supplementary Material, Figure S3A.

Replication of the GWAS in subjects of Native American ancestry

To replicate our findings we performed a second GWAS on subjects of Native American descent (NAs), including subjects with varying degrees of NA admixtures as typically seen in Hispanic subjects ($n = 91$). The mean pDBH level in 93 NAs was 10.2 IU/l (SD = 6.94) and was not significantly different from pDBH levels in EAs ($P > 0.29$). The genomic control inflation λ_{GC} was 1.009 (a QQ-plot is shown in Supplementary Material, Fig. S2B). A table with all GWAS results is available in the Supplementary Material, Table S2. Replicating our results in EAs, we confirmed the *DBH* locus to be highly significant, with the same top hit rs1611115 at $P = 4.10 \times 10^{-15}$ (Table 1 and Supplementary Material, Fig. S4A). A regional association plot of the *DBH* locus showed an additional five intronic genome-wide significant SNPs within the same LD-block (Supplementary Material, Fig. S4B). The proportion of variability explained (R^2) by the *DBH* locus, based on four independent (LD < 0.5), highly significant *DBH* SNPs (including the three promoter SNPs), was 0.57.

We did not identify other genome-wide significant regions in this small NA population. Eight other loci showed suggestive evidence for association with pDBH activity ($P < 5 \times 10^{-6}$). For each of these regions the SNP with the lowest *P*-value is presented in Table 1 (middle part) and Supplementary Material, Figure S3B.

Meta-analysis of EA and NA GWAS

An inverse variance weighted meta-analysis of the EA and NA GWAS results indicated no significant heterogeneity (*Q*) at the *DBH* locus and resulted in highly significant associations for the promoter region of this locus with the top hit rs1611115 at $P = 4.60 \times 10^{-92}$, as well as rs1076150 (T-2734C) and rs1989787, at $P = 1.38 \times 10^{-44}$ and $P = 1.50 \times 10^{-34}$, respectively (Table 1, right side and Supplementary Material, Fig. S5A). A complementary pooled analysis (mega-analysis) of the EA and NA subjects for the three promoter SNPs showed comparable results (Supplementary Material, Fig. S5B). A C to T transition progressively diminished pDBH activity for rs1076150 and rs1611115, while increasing pDBH activity for rs1989787. In each case, SNP allele effects on trait seemed to be additive, with intermediate effects for SNP heterozygotes, confirmed by the fact that recessive and dominant genetic models were less significant than the additive model for these three SNPs (data not shown).

In addition to the *DBH* locus, the meta-analysis showed a genome-wide significant association for intronic SNP rs4255618 in *LOC338797* on chromosome 12 ($P = 4.62 \times 10^{-8}$). A BLAST search (on BLASTN_2.2.28+ at NCBI) of the RNA-coding region (~20 kb) of this uncharacterized locus showed no homology to *DBH*. In addition, seven new loci reached suggestive evidence for association in the meta-analysis (top hits for these loci are shown in Table 1). The proportion of variability explained (R^2) by the *DBH* locus, based on seven highly significant *DBH* SNPs in low LD with each other (including the three promoter SNPs), was 0.57. Adding rs4255618 in *LOC338797* to the *DBH* model significantly increased R^2 to 0.59 (LR test $P = 6.09 \times 10^{-5}$) in a joint analysis of EA and NA subjects.

Conditional analysis on the *DBH* locus

Because of the strong effect of the *DBH* locus on pDBH activity, we repeated the GWAS conditioned on the three *DBH* peak functional promoter SNPs rs1076150, rs1989787, and rs1611115 in EAs, NAs and the meta-analysis to test for additional, *DBH*-independent loci (Supplementary Material, Fig. S6). The *SARDH* locus, adjacent to *DBH* and previously showing suggestive evidence for association, became genome-wide significant in EAs with an imputed top hit for rs7857468 ($P = 2.38 \times 10^{-13}$). Rs7857468 replicated in NAs with a nominally significant $P = 0.002$, resulting in a meta-analysis *P*-value = 1.15×10^{-16} (Table 1, bottom part). Results for the most significant directly genotyped SNP in *SARDH* (rs7040170, $P = 1.31 \times 10^{-14}$) are also shown. Regional association plots of the conditioned GWAS results in EAs and NAs for the *DBH* and neighboring *SARDH* loci are shown in Supplementary Material, Figure S6B and D. Adding the *SARDH* SNP to the *LOC338797* and *DBH* model significantly increased R^2 to 0.648 (LR test $P = 8.13 \times 10^{-16}$) in a joint analysis of EA and NA subjects. The conditional analysis did not result in stronger results for the loci showing suggestive evidence in the primary analyses (Supplementary Material, Fig. S6A and C).

Functional analysis of variant C-2734T and four naturally occurring haplotypes in the *DBH* promoter

Functional analyses of the promoter variants rs1611115 and rs1989787 have previously been published by our group (14,15). Here we extend these analyses to the third promoter variant rs1076150, identified in the GWAS with a highly significant effect. Using the same six common promoter SNPs (minor allele frequency MAF > 0.05) as in previous work, we constructed luciferase promoter plasmids for four common, naturally occurring six-SNP haplotypes from the BAC promoter insert. The promoter activity of these four natural haplotypes (HAPs 1–4), measured as a function of luciferase expression in chromaffin cells is shown in Supplementary Material, Figure S7. We found that genotypic variations showed a significant overall effect ($F = 33.8$, $P < 0.001$), with haplotypes showing different *DBH* promoter/luciferase reporter activities (expressed as Firefly/Renilla ratio). To evaluate the individual effect of the rs1076150 SNP we constructed mutant variants on balanced backgrounds for two of the four haplotypes (HAP2 and HAP4), differing only at the desired −2734 position. When compared with the T allele, the C allele displayed higher expression on two different backgrounds (HAP2: $P = 0.0047$ and HAP4: $P = 0.0098$) (Fig. 2).

Bioinformatics of variant promoter motifs

In order to further investigate the functional properties of the *DBH* promoter variant rs1076150, we used bioinformatics tools (CONSITE and MotifLab) for the analysis of regulatory sequences. Both tools predicted that at position −2734 (upstream from the translation start site), SNP rs1076150 disrupted a binding motif for the transcription factor Snai1. As indicated in Supplementary Material, Figure S8, the match and binding score for the C-allele were predicted to be higher than for the T allele, possibly resulting in different expression levels of the DBH protein. For a complete characterization of the DBH promoter region, the computational molecular predictions and proposed mechanistic consequences of disrupted transcription factor binding motifs for the other two functional promoter variants rs1611115 and rs1989787 were added in Supplementary Material, Figure S8.

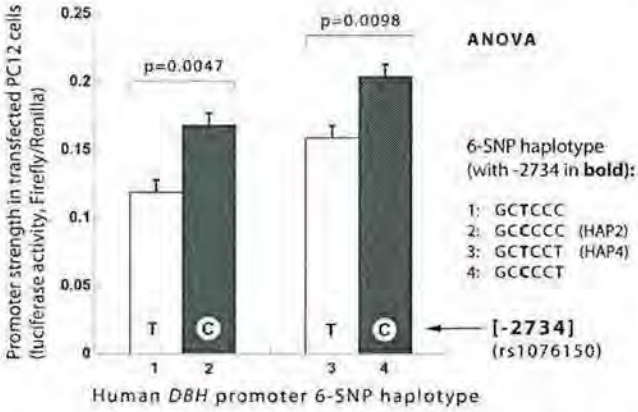

Figure 2. *In vitro* effects of human *DBH* promoter variant C-2734T (rs1076150): Balanced mutants on two haplotype backgrounds (HAP2, HAP4) yield consistent (C > T) effects on transcription in chromaffin cells. Strength of the promoter variants is shown as luciferase activity in PC12 cell type (mean ± SEM). *P*-values are result of C versus T variant comparison for each haplotype background by ANOVA.

In vivo effects of functional *DBH* promoter haplotypes on human pDBH activity

We further evaluated the directional effects of the three functional SNPs (rs1076150 → rs1989787 → rs1611115) in the *DBH* promoter region (which showed the highest associations with pDBH activity in the GWAS) in a haplotype analysis in the combined 434 EA and NA subjects. First, we considered haplotype homozygotes for the four naturally occurring diploid haplotypes (Fig. 3A), and noted significant differences in pDBH activity with a plasma activity rank order of: CTC>CCC>TCC>TCT ($P = 1.84 \times 10^{-29}$). Finally we analyzed the effects of haplotype copy number on pDBH activity for the four haplotypes (Fig. 3B). The results were internally consistent with those for haplotype homozygotes, showing that increasing CTC copy number progressively elevated pDBH activity ($P = 7.49 \times 10^{-32}$), with reciprocal effects for haplotype TCT copy number ($P = 2.96 \times 10^{-66}$). Corresponding individual SNP effects are also shown in Supplementary Material, Figure S5A.

Application of the MR test using genetic variants in DBH

PTSD re-experiencing symptoms were assessed post-deployment in 402 subjects with available pDBH levels and ranged from 0 to 29 (mean = 5.87). Re-experiencing symptoms were significantly associated with pDBH (beta = 0.13, $P = 0.012$), making a MR analysis applicable. The MR estimate of the association of pDBH and re-experiencing symptoms was significant (beta = 0.21, $P = 0.002$), indicating that pDBH is a causal component in the development of re-experiencing symptoms.

DISCUSSION

Dopamine β-hydroxylase as an essential part of the catecholamine biosynthetic pathway, converts dopamine to norepinephrine. DBH is encoded by a single gene located on chromosome 9q34 and its enzymatic activity is expressed both in plasma

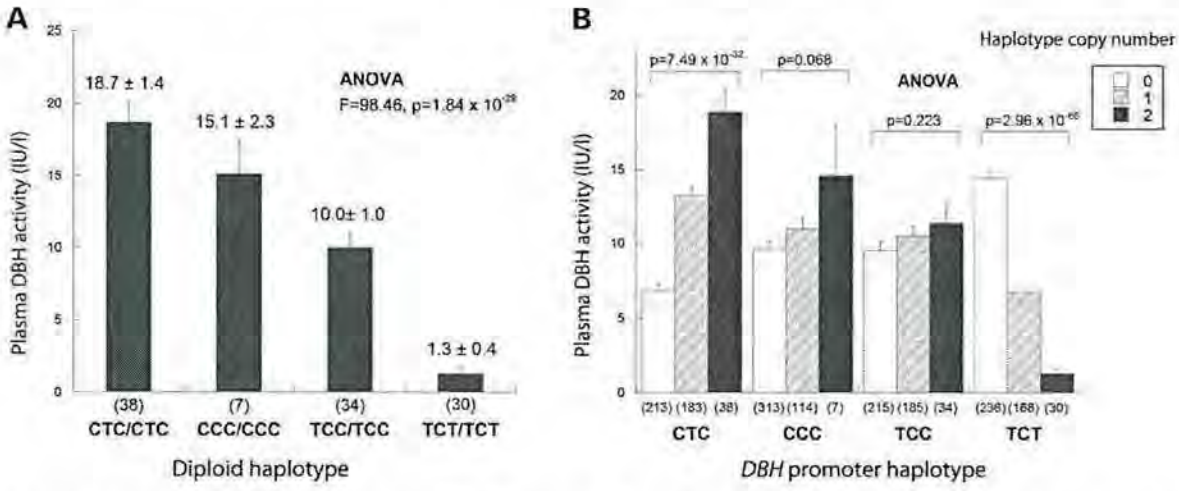

Figure 3. *In vivo* effects of *DBH* promoter functional variants T-2734C (rs1076150), C-2073T (rs1989787) and C-970T (rs1611115) on plasma DBH activity (IU/l). (**A**) *DBH* promoter diploid haplotype (rs1076150 → rs1989787 → rs1611115) effect on pDBH activity (IU/l). Only subjects homozygous for a given haplotype (rs1076150 → rs1989787 → rs1611115) are shown. (**B**) Effect of *DBH* promoter haplotype (rs1076150 → rs1989787 → rs1611115) copy number (0, 1, or 2 copies per genome) on pDBH activity (IU/l, adjusted mean ± SEM).

and CSF. The effects of this *cis*-QTL on plasma, serum and/or CSF DBH activity have been previously investigated in isolation (14,22,24), but to date no genome-wide association studies have been reported on DBH activity. Here, we present the first GWAS of plasma DBH levels and further characterize transcriptional control of the *DBH* gene.

Our GWAS was first performed in subjects of EA ancestry. We replicated the *DBH* locus as major contributor to pDBH activity, explaining ~57% of the variability in EAs. As found by others, rs1611115 was the most significant polymorphism in this gene (22), with a $P < 7.2 \times 10^{-51}$, by far exceeding the genome-wide significance threshold of $P < 5 \times 10^{-8}$, and another 33 SNPs (some of them with independent effects) at this locus met genome-wide significance. No other loci were found to be genome-wide significant in this relatively small sample of 341 EAs, but 10 loci reached suggestive evidence of association with pDBH at $P < 5 \times 10^{-6}$ and await further replication in larger datasets. However, none of these loci were located on 20p12, a *trans*-QTL suggested in a linkage study by (24). The often poor correspondence between the susceptibility loci identified in genetic linkage and genome-wide association studies may be due in part to allelic heterogeneity, which reduces power in GWAS compared to linkage analyses (27).

Genetic association studies on the DBH locus have compared the three main ancestry groups from Europe, Africa and Asia. EAs were reported to have higher mean pDBH levels as compared to Japanese (22) and Africans from Nigeria (14,25). The promoter SNP rs1611115 was consistently reported as the most significant candidate SNP in *DBH* across studies and ancestral groups (14,22,23,28). Here, we extend this work to include subjects of genetically determined Native American descent, typically self-identifying as either Native American or Hispanic in our study. We found no difference in pDBH activity levels between our EA and NA subjects. The GWAS replicated the DBH locus with the same top hit (rs1611115 at $P = 4.1 \times 10^{-15}$) and consistent effect size estimates ($R^2 = 0.59$ and 0.57 in EAs, respectively) in this even smaller sample of 93 subjects.

Increasing our power to detect additional loci by combining the relatively small number of EA and NA subjects in a meta-analysis, we identified *LOC338797* (rs4255618) on chromosome 12q at $P = 4.62 \times 10^{-8}$, meeting the traditional genome-wide significance threshold of 5×10^{-8}. However, genotype imputations based on 1000 Genomes Project reference data are increasing the effective number of independent tests and more stringent thresholds have recently been suggested (e.g. 1×10^{-8} for all common SNPs) (29). Irrespective of the specific threshold selected, the relevance of *LOC338797* and all findings showing suggestive evidence of association have to be confirmed through independent replication of these results. *LOC338797* seems to encode a 4-exon, previously uncharacterized 1794-base lncRNA, but the RNA-coding region bears no homology to *DBH* itself, and its role in DBH remains to be determined. However, adding *LOC338797* to our genetic model of DBH only marginally increased the percent trait variability explained (from 57 to 59% in the combined analysis).

An additional analysis conditioned on the *DBH* locus promoter SNPs, to mask its strong effect on trait, identified sarcosine dehydrogenase *SARDH*, a gene adjacent to *DBH*, as an apparently independent, genome-wide significant hit in EAs. Its top hit rs7857468 was nominally replicated in NAs, leading to an overall *P*-value of 1.15×10^{-16}, and further improving our model to explain 65% of overall variability in pDBH activity. *SARDH* encodes an enzyme localized to the mitochondrial matrix that catalyzes the oxidative demethylation of sarcosine. Even though adjacent to (and within 86.6 kb of) DBH, the conditional peak *SARDH* markers displayed little LD with the DBH promoter, as judged by marker-on-marker LD ($R^2 < 0.2$) as well as a cM/Mb recombination boundary peak (Supplementary Material, Fig. S6B and D). However, analysis of the local chromosomal region by Chromatin conformation capture (or Hi-C, (30)) in human ES cells as well IMR-90 fibroblasts revealed that both *DBH* and *SARDH* inhabit the same topological domain, bounded by insulator/barrier (CTCF motif) elements. Thus, it is conceivable that the *SARDH* region harbors a 3′ transcriptional enhancer for *DBH* expression.

Mechanisms underlying DBH expression and secretion into plasma and CSF have invoked continuing interest among a broad range of investigators. One genetic variant in particular (rs1611115) has been widely investigated and ultimately documented (14) as a functional variant in the *DBH* promoter (14,22). We previously conducted systematic polymorphism discovery across the human *DBH* locus, and probed the functional consequences of two promoter variants (rs1989787 and rs1611115). We showed that rs1611115 disrupted consensus transcriptional motifs for *n-MYC* and *MEF-2* (14) and rs1989787 for *c-FOS* (15), and that trans-activation of these variants by the corresponding transcription factors resulted in changes in *DBH* expression. The effects of variant rs1076150 on transcription reported here are novel, and allowed us to evaluate the effects upon gene expression of all three functional variants simultaneously. Here, we present an overview of properties of all three major functional variants in the proximal *DBH* promoter (Supplementary Material, Fig. S8). We found additive effects of each functional SNP upon DBH secretion into plasma (Supplementary Material, Fig. S5), and noted that the activity of contributory SNP alleles summated to give rise to a spectrum of promoter haplotype activities (Fig. 3A and B).

Genetic variants in *DBH* and/or pDBH activity have been directly implicated in mechanisms leading to increased susceptibility to disease. As the final enzyme in norepinephrine biosynthesis, DBH plays a role in differential availability of dopamine and norepinephrine. Consequently, DBH is involved in mechanisms underlying disorders associated with changes in the noradrenergic system (31–35). For example, our most significant *DBH* variant (rs1611115) is influencing heritable 'intermediate phenotypes' (e.g. autonomic and renal traits) as physiological risk traits in later development of hypertension (e.g. the T allele was found to decrease urine epinephrine excretion and basal blood pressure) (14,15) and progressive renal disease (36). In addition, biological and genetic studies suggest associations of low DBH levels with psychotic symptoms, and with mental disorders such as schizophrenia, depression, attention deficit hyperactivity disorder and alcoholism (see review 16). However, large GWAS on cardiovascular and psychiatric disorders (e.g. as reported by Ricopili) did not replicate strong effects for genetic variants in *DBH*.

The large proportion of DBH heritability that can be explained by a small number of genetic markers, in combination with the potentially important role of this intermediate phenotype for both psychiatric and cardiovascular disorders is unique and

may represent a useful methodological tool to develop and test genetic epidemiological methods (37,38). To this end, we have applied genetic markers in *DBH* to the MR approach to investigate a potential causal effect of the pDBH and PTSD association previously reported (12,13). Our preliminary results on the effect of pDBH on PTSD re-experiencing symptoms indeed support this causal relation, but these findings will need to be confirmed in larger studies.

In conclusion, a first GWAS on pDBH activity identified the *DBH* gene as the principal locus determining pDBH levels in both EA and NA populations, explaining 57% of the variability. Two additional novel loci, *SARDH* and *LOC338797*, explaining combined an additional 8% of overall variability, were identified here and will have to be replicated in independent studies. Compared with other GWAS studies, the effects reported here were detected in relatively small datasets. Future studies on larger datasets may discover additional loci of smaller effects. Further, we demonstrated the potential application of strong genetic predictors of intermediate phenotypes such as DBH to the investigation of the disease etiology in the context of PTSD.

In perspective, the characterization of DBH activity and its underlying genetic regulation has positioned us uniquely for future studies of 'intermediate phenotypes', potentially leading to discovery of causal variants in complex genetic traits and disorders such as found in the psychiatric and cardiovascular fields.

MATERIALS AND METHODS

Subjects and biological sample collection

Participants were recruited from the Marine Resiliency Study (MRS), a large, prospective study of post-traumatic stress disorder (PTSD) involving active-duty United States Marines bound for deployment to Iraq or Afghanistan (39). The protocols for these studies were approved by the University of California-San Diego Institutional Review Board (IRB Protocols #070533, #110770X), and all subjects provided written informed consent to participate. Here we evaluated a subgroup of the MRS with available genotype and pDBH activity phenotype data, including 532 healthy, unrelated males from four different battalions (cohorts) assessed at pre-deployment. Following a 7-month deployment to a combat zone, post-traumatic stress symptoms were evaluated using a structured diagnostic interview, the Clinician Administered PTSD Scale (CAPS; (40–43)). Inter-rater reliability in MRS for the CAPS total score was high (Intraclass correlation coefficient = 0.99). Re-experiencing symptoms (CAPS-B symptom cluster) were used here. Initially, ethnicity and race were established by self-report, including information on geographic origin of both parents. The cohort studied here included 86% Caucasian and 22% Hispanic subjects, with a mean ($\pm$SD) age of 22.41 $\pm$ 3.23 years (range 18–41), typical for the overall MRS participants.

Blood was sampled from an antecubital vein for preparation of heparinized plasma (for assay of pDBH activity) and EDTA-anticoagulated blood (for preparation of genomic DNA). Heparinized blood from lithium heparin tubes was kept on ice prior to centrifugation and plasma was stored at -70°C prior to thawing for assays in batch. Genomic DNA was prepared from 1–2 ml blood leukocytes and diluted to a standard concentration of 50 ng/μl for genotyping.

Genotyping, quality control procedures and genotype imputations

Genotyping of 2585 DNA samples (532 with pDBH activity measures) was carried out by Illumina (http://www.illumina.com/) using the HumanOmniExpressExome array (HOEE 8v1_A) with 951 117 loci. Initial allele calling was performed by Illumina in GenomeStudio (V2011.1) and resulted in a sample success rate of 99.65%, a locus success rate of 99.86%, a genotype call rate of 99.88%, with reproducibility including 28 replicate DNA sample pairs of >99.99%. Additional data cleaning was performed in PLINK v1.07 (44) using standard procedures. SNPs were excluded if the call rate was <95%, if they violated Hardy–Weinberg Equilibrium ($P < 1 \times 10^{-6}$), or if they showed plate effects (P-value $< 1 \times 10^{-8}$ for any one plate or $< 1 \times 10^{-4}$ for two or more plates). Sample ID was confirmed by evaluating concordance between 31 overlapping genotypes from the HOEE array and those from an initial 'fingerprinting' panel including 41 ancestry-informative markers (AIMs) (45), resulting in the exclusion of one sample (overall concordance rate >0.99). Unexpected familial relationships were identified using pairwise identical-by-descent estimation and two subjects from sib-pairs were removed. Sample heterozygosity was between 0.211 and 0.302 and no excessive high or low samples were identified. The final dataset included 851 541 markers genotyped in 2548 individuals with a genotyping rate of >0.998.

Imputations were performed with standard protocols using the default parameters in IMPUTE2 v2.2.2, using 1000 Genomes Phase 1 integrated variant set haplotypes for the autosomes and the interim set for the X chromosome. Prior to imputation, genetic markers that had exceedingly rare alternative alleles (minor allele frequency MAF < 0.0002) were excluded. Next, genomes were divided into ~5 Mb segments, and phasing and imputed genotypes were calculated for each. Imputed markers with low imputation quality values (Info value $\leq$0.5) were excluded. GTOOL v0.7.0 was used to convert genotype probabilities into calls for markers with probabilities >90% (genotypes were called missing if the posterior probability of any genotype was $\leq$90%), resulting in a total of 24 068 319 successfully imputed polymorphic markers, and a total of 24 919 860 genotyped and imputed markers for association analyses.

Ancestry assessment and control for genetic background heterogeneity

Ancestry was determined using genetic information as described in (45). In brief, genotypes of 1783 AIMs were used to determine a subject's ancestry at the continental level for the seven geographic regions Africa, Middle East, Europe, Central/South Asia, East Asia, Americas and Oceania. Ancestry estimates were determined using STRUCTURE v2.3.2.1. (46) at $K = 7$, including prior population information of the HGDP reference set (47). Based on these ancestry estimates, MRS subjects included here were placed into two main ancestral groups: subjects with >95% European ancestry were grouped with EAs ($N = 341$); and subjects with >5% Native American ancestry (and <10% African, and <5% each Central Asian, East Asian and Oceanic ancestry) as Native American descendants (NAs) ($N = 93$). A very wide range of Native American ancestry

proportions is typical for subjects of self-reported Hispanic and Native American ethnicity/race (e.g. (48,49). Subjects with other ancestral backgrounds were not analyzed here ($N = 98$).

GWAS was performed separately in 341 EAs and 93 NAs. To control for additional genetic background heterogeneity within the two ancestral groups, and varying degrees of EA admixture within the NAs, principal component analyses (PCA) implemented in the EIGENSTRAT software (50) based on 10 000 random, autosomal SNPs were performed. The first 3 Eigenstrat-derived PCAs were included each as covariates in the association analyses.

Functional effects of trait-associated *DBH* promoter variants (rs1076150, rs1989787, rs1611115): promoter/luciferase reporter activity assays

Human *DBH* promoter/reporter plasmids were constructed from BAC genomic clone (RP11-317B10) obtained from CHORI (http://bacpac.chori.org) as described before. The *DBH* promoter region (extending distally from −3000 to +51 bp) containing six common polymorphic sites was excised from the BAC clone and inserted into the upstream/polylinker region of firefly luciferase reporter plasmid pGL3-Basic (Promega; Madison, WI, USA). Common naturally occurring haplotypes and additional variants were made by site-directed mutagenesis (QuikChange, Stratagene (Agilent), Santa Clara, CA, USA), verified by dideoxy sequencing, and co-transfected with Renilla luciferase expression plasmid pRL-TK (Herpes simplex virus thymidine kinase promoter driving Renilla luciferase, Promega) as a transfection efficiency control, into PC12 pheochromocytoma cells (at ~50–60% confluence, 1 day after 1:4 splitting) as previously described (14). Firefly and Renilla luciferase activities in cell lysates were measured 16 h post-transfection, and results were presented as Firefly/Renilla luciferase activity ratio ('Stop & Glo'; Promega, Madison, WI, USA).

Biochemical properties of plasma DBH

Plasma DBH activity was measured in 25 μl of heparinized plasma by a modified Nagatsu/Udenfriend spectrophotometric method (51), and reported as IU/l (IU/l=μmol/min/l plasma at 37°C, protocol available online at http://hypertension.ucsd.edu/). This method is based on a conversion of the synthetic DBH substrate tyramine by DBH (in the presence of Cu^{2+}, N-ethylmaleimide and fumarate) to octopamine, which is then oxidized to parahydroxybenzaldehyde by sodium periodate. The oxidation is terminated by sodium metabisulfite, and the end product parahydroxybenzaldehyde is quantified by its absorbance at 330 nm in the ultraviolet spectrum. The mean plasma DBH activity inter-assay coefficient of variation was 12.8%. The mean plasma DBH level in 532 subjects was 10.86 IU/l (SD = 6.77) and ranged from 0.01 to 37.41 IU/l (Supplementary Material, Fig. S1).

Bioinformatic analyses

Computational prediction and motif discovery for transcription factors in the promoter region of *DBH* where candidate SNPs were positioned was made using web interface tools CONSITE (52) and graphical interface MotifLab (53), available at (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/) and (http://tare.medisin.ntnu.no/motiflab/), respectively. For both tools, predictions were based on position weight matrices for binding sites annotated in JASPAR and TRANSFAC databases. Motifs from consensus sequences, whose score was higher than 80% for binding to a motif containing a target SNP, were considered candidates.

Statistical analyses

Plasma DBH levels were square-root transformed to conform to normality ($P > 0.74$, Kolmogorov–Smirnov test). GWAS of transformed plasma DBH levels was performed in EA ($N = 341$) and NAs ($N = 93$) separately using linear regression under an additive genetic model with covariates age, cohort (three dummy coded variables), and three PCAs as implemented in PLINK. SNPs were pruned to a minor allele frequency (MAF) ≥ 0.01 in the combined dataset, which resulted in the inclusion of 7 871 575 SNPs. Genome-wide significance was set to $P < 5 \times 10^{-8}$ and suggestive evidence for association was considered at $P < 5 \times 10^{-6}$. Meta-analyses on the EA and NA results were performed in PLINK, using a fixed-effects model for SNPs with no significant heterogeneity (I) and a random-effects model when heterogeneity was significant (Cochrane's Q statistic). Conditional analyses on the *DBH* locus were performed to identify additional genetic associations by including the three *DBH* peak promoter SNPs rs1076150, rs1989787 and rs1611115 as additive covariates. Percent variability explained (R^2) by a SNP or multiple SNPs in a gene were calculated using a linear regression in R 3.0.0, using the—clump function in PLINK to generate a list of highly significant SNPs in low LD for each gene with genome-wide significant SNPs. QQ plots and Manhattan plots were made using R 3.0.0. LocusZoom 1.2 (54) was used to construct regional association plots, including recombination information from HapMap phase II CEU. SG-ADVISER (http://genomics.scripps.edu/ADVISER/) was used for SNP annotations.

Analysis of variance (ANOVA) was used to compare luciferase reporter activity between different *DBH* haplotypes *in vitro*, and linear regression models and ANOVA based on an additive genetic model, with age, cohort and three PCAs as covariates were used for *in vivo* experiments to test for associations of haplotypes with DBH enzymatic activity in plasma using IBM SPSS Statistics, v.20.

Associations between pDBH levels, CAPS total score and symptom cluster B were tested in the combined EA ($N = 341$) and NA ($N = 93$) sample. To account for the non-normal distribution of CAPS scores, a zero-inflated negative binomial distribution (ZINB) regression was used (55), with additional covariates age, cohort (three dummy coded variables), and three PCAs based on continental ancestry. Associations between *DBH* SNPs and CAPS scores were tested under an additive genetic model.

Instrumental variable analysis. To demonstrate the utility of strong genetic effects on intermediate phenotypes for application to a MR approach, an association of pDBH with post-deployment PTSD re-experiencing symptoms was tested, using a ZINB regression (55), with additional covariates age, cohort and PCs. Following the determination of a significant

association, the DBH SNP with the strongest effect (rs1611115) on pDBH was used as an instrument to test if pDBH is in the causal pathway to disease development (i.e. PTSD). MR estimates for the effect of pDBH on CAPS were then derived using a control function approach (56) an ordinary least squares regression of pDBH levels on rs1611115 was performed, including covariates age, cohort and PCs, followed by a ZINB regression of the CAPS score on pDBH, including the residuals from the first regression and age, cohort and PCs as covariates.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Chip-based direct genotyping of coding variants in genome wide association studies: Utility, issues and prospects

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ABSTRACT

There is considerable debate about the most efficient way to interrogate rare coding variants in association studies. The options include direct genotyping of specific known coding variants in genes or, alternatively, sequencing across the entire exome to capture known as well as novel variants. Each strategy has advantages and disadvantages, but the availability of cost-efficient exome arrays has made the former appealing. Here we consider the utility of a direct genotyping chip, the Illumina HumanExome array (HE), by evaluating its content based on: 1. functionality; and 2. amenability to imputation. We explored these issues by genotyping a large, ethnically diverse cohort on the HumanOmniExpressExome array (HOEE) which combines the HE with content from the GWAS array (HOE). We find that the use of the HE is likely to be a cost-effective way of expanding GWAS, but does have some drawbacks that deserve consideration when planning studies.

1. Introduction

Methods to extend genome-wide association studies (GWAS) have recently become a topic of high interest. Despite a large number of notable successes in the discovery of genetic variants associated with various traits, including disease via GWAS, the variants identified to date collectively only explain a small fraction of the estimated heritability of most common, chronic diseases (Manolio et al., 2009). Unknown genetic factors, including polymorphisms that have yet to be identified through GWAS studies, likely account for the 'missing heritability' associated with complex traits (Visscher et al., 2012; Yang et al., 2011). One explanation for this missing heritability is that widely-used genotyping platforms for GWAS are designed to directly interrogate only common single nucleotide polymorphisms (SNPs). Therefore, rare coding variants, which have been shown to play a role in the etiology of many diseases, tend to be entirely omitted by most genotyping platforms used in GWAS as they are not in linkage disequilibrium (hence not imputable) with SNPs interrogated on these arrays (Evans et al., 2008; Sun et al., 2011). Thus, the examination of rare coding variants requires either sequencing technology or the direct genotyping of variants which have previously been identified. While the former may lead to a more comprehensive assessment of all forms of variation in coding regions, including the discovery of extremely rare and/or *de novo* variants, the latter provides an efficient, cost-effective alternative for interrogating a subset of known variants in coding regions (Flannick et al., 2012; Pasaniuc et al., 2012).

The value of direct genotyping of previously identified coding variants, as opposed to *de novo* sequencing of coding regions, is dependent on a few key issues. First, if one can identify known functionally relevant variants in coding regions it might be more expedient to focus on them in cost-effective direct genotyping studies than pursuing more costly

Abbreviations: HE, HumanExome array; HOEE, HumanOmniExpressExome array; HOE, HumanOmniExpressGWAS array; GWAS, genome-wide association studies; SNPs, single nucleotide polymorphisms; MRS, the Marine Resiliency Study; PTSD, post-traumatic stress disorder; OEF/OIF, Operation Enduring Freedom/Operation Iraqi Freedom; IRB, Institutional Review Board; HGDP, Human Genome Diversity Project; MAF, minor allele frequency; SNVs, single-nucleotide variants.

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sequencing studies that may identify many likely neutral variants. Second, if coding variants identified via sequencing are easily imputable from variants genotyped on standard GWAS platforms, then the need for directly genotyping these coding regions would be minimized and greater attention could be given to more reliable imputation strategies. Third, many coding variants, whether they are functional or amenable to imputation or not, are very rare and hence likely to be absent in many global populations. Thus, direct genotyping certain coding variants may only be useful for specific populations.

Here we assessed the potential benefits of directly genotyping rare coding variants on the Illumina Human Exome (HE) array by addressing these issues. As such, our assessment includes an examination of the functional content of variants included on the array. We also evaluated the amenability of the HE markers to imputation from the Illumina Human Omni Express (HOE). And lastly, we evaluated the allele frequency spectrum of the variants included on the HE chip. We find that, overall, the HE chip does not suffer severe drawbacks in the context of these issues, but of course is limited to assessments of known (i.e., previously identified) variants. Our analyses and results have important implications for future studies seeking to identify associations with coding variants.

2. Material and methods

2.1. Subjects and genotyping

Participants were recruited from two southern Californian military personnel cohorts: 1. the Marine Resiliency Study (MRS), a prospective study of post-traumatic stress disorder (PTSD) involving United States Marines bound for deployment to Iraq or Afghanistan (Baker et al., 2012); and 2. a cross-sectional study of active duty service members and veterans of Operation Enduring Freedom/Operation Iraqi Freedom (OEF/OIF) (Pittman et al., 2012). The protocols for these studies were approved by the University of California-San Diego Institutional Review Board (IRB Protocols #110770, #070533, and #080851), and all subjects provided written informed consent to participate.

DNA samples from 2585 study participants were acquired, and genotyping was carried out by Illumina (http://www.illumina.com/) using the HOEE version 12v1.0. Initial allele calling was performed by Illumina in Genome Studio (http://www.illumina.com) and the overall data quality was high: sample success rate was 99.95% (9 samples failed), locus success rate was 99.86%, and genotype call rate was 99.88%. Twenty-eight replicate pairs of samples undergoing genotyping were assessed for consistency and ultimately reproducibility of the assay and agreement of genotyping calls was achieved for >99.99% over all genotypes across these 28 pairs. Additional data cleaning was performed in PLINK v1.07 (Purcell et al., 2007) and included the removal of 224 markers with heterozygous haploid genotypes on the X, Y, or mitochondrial chromosome. The final dataset included 949,469 markers genotyped in 2548 individuals (2538 males and 10 females) with a genotyping rate greater than 99.8%.

2.2. Ancestry determination

We estimated each individual's degree of European, African, Native American, Central Asian, East Asian and Oceanic admixture by comparing the individual's genotypes to allele frequencies of 10,079 SNPs in common with a large set of reference individuals (Libiger and Schork, 2013). In short, the reference sample consisted of genotype data for 2513 individuals of known ancestry who originated from 83 populations from around the world. These data were assembled from publicly available sources including the Human Genome Diversity Project (HGDP) (Cann et al., 2002), the Population Reference (POPRES) (Nelson et al., 2008), HapMap3 (Altshuler et al., 2010), and the University of Utah dataset (Xing et al., 2009). Admixture estimates were obtained in two steps using a supervised analysis implemented in the ADMIXTURE software (Alexander et al., 2009). In the first step, we computed initial admixture estimates for all individuals associated with each world population using the entire set of reference individuals and determined the estimates' standard errors via bootstrapping. A subset of reference individuals from populations that exhibited evidence of contributing to an individual's ancestry based on 95% confidence intervals was then used to refine the initial admixture estimates in a subsequent supervised ADMIXTURE analysis.

Final ancestry calling was based first on self-reported race and ethnicity information and second within each of these main population groups. Essentially, subjects were placed into 5 groups: European Americans (subjects with >95% European ancestry; N =1476), Asian Americans (>95% East Asian ancestry; N = 43); African–American (subjects with >5% African ancestry and <5% Native American, Central Asian, East Asian and Oceanic ancestry; N = 109), Hispanic Americans (subjects with >5% Native American and <10% African, Central Asian, East Asian and Oceanic ancestry; N = 321), and Other (all others; N = 599). Thus, our ancestry assignments provide initial assignments consistent with the often-used admixture program except that they have been refined by removing noise and leveraging comparisons to self-reported ancestries.

2.3. Genotype imputations

Imputations were conducted using markers available on the HOE platform. Prior to imputation, mitochondrial and unmapped SNPs were removed from each set. Markers that were individually rare (minor allele frequency MAF < 0.0002), showed a large number of missing genotypes (>5%), or failed Hardy–Weinberg equilibrium ($p < 1 \times 10^{-6}$) were also removed (Supplemental Table 1). Imputations were performed using the default parameters in IMPUTE2 v2.2.2, using 1000 Genomes Phase 1 integrated variant set haplotypes for the autosomes and the interim set for the X chromosome (Howie et al., 2009). IMPUTE2 is well suited for imputations on genetically diverse and admixed populations such as that of the present study as the algorithm is robust to ancestral genetic variation within the reference panel and study datasets (Howie et al., 2011). Genomes were divided into approximately 5 Mb segments (minimum 2.5 Mb, maximum 7.5 Mb to avoid chromosome and centromere boundaries), and phasing and imputed genotypes were calculated for each. Imputed markers with low imputation quality values (Info ≤ 0.5) were dropped. GTOOL v0.7.0 was used to convert genotype probabilities into calls. Individual genotype probabilities exceeding 90% were assigned genotype calls and probabilities ≤90% were treated as missing genotypes. Agreement between the imputation results and markers exclusive to HOEE (i.e., HE markers) was examined by calculating the correlation coefficient, r^2, between calls on a per marker level. Missing genotypes were assigned an allelic dosage representing the mean genotype at that particular locus for all calculations. Imputation was also performed based on genotype data from the HOEE platform. A comparison of the agreement between the HOE and HOEE to impute markers that were not genotyped on either platform was, likewise, conducted.

2.4. Variant functional annotations

We mapped all variants to the closest gene from the UCSC Genome Browser known gene database (Fujita et al., 2011). Full details of our annotation pipeline are described in a previous publication (Torkamani et al., 2012) and the Supplemental Methods. In brief, variants were associated with all transcripts of the nearest gene(s), with functional impact predictions made independently for each transcript. If the variant fell within a known gene, its position within gene elements (e.g. exons, introns, untranslated regions, etc.) was recorded for functional impact predictions depending on the impacted gene element. All variants falling within an exon were analyzed for their impact on the amino acid sequence (e.g. synonymous, nonsynonymous, nonsense, frameshift, in-frame, intercodon etc.).

3. Results

3.1. Characterization of the cohort

Table 1 provides a description of the cohort based on self-reported race and ethnicity information and includes the number of subjects, gender, and age of the subjects and the number of individuals removed from the study because of failed genotyping quality control (see Methods). Individual ancestry and admixture proportions were assessed within these self-reported race and ethnicity groups using genotype information (see also Methods) and a graphical representation of the ancestry/admixture among the subjects in the study is provided in Fig. 1. We ultimately identified 1476 individuals with predominantly European ancestry, 109 African–American individuals, 43 with predominantly East Asian ancestry, 321 with predominantly Hispanic American ancestry (i.e., with significant Native American admixture), and 599 with predominant ancestry from any other geoethnic population. We used these combined self-reported and genetically-determined ancestries in subsequent analyses.

3.2. Imputability of the HE markers

We explored the possibility that the markers which were exclusive to the HOEE array (i.e., the HE content) could be imputed from markers on the HOE array. If these markers are amenable to imputation, it would call into question the utility of the additional content on the HOEE chip. Only a modest proportion of the markers exclusive to the HOEE array were imputable from the HOE content and passed imputation quality control thresholds (N = 80,205; 32.9%). Among these, markers with common variants (MAF > 0.05; N = 27,250) were imputed accurately across all ethnicities: 76.4% of common markers had $r^2 > 0.95$ and 90.6% had $r^2 > 0.80$. However, markers with moderately common ($0.01 \leq MAF \leq 0.05$; N = 9777) and rare (MAF < 0.01; N = 43,178) variants were imputed more poorly: 46.8% and 22.9% with $r^2 > 0.80$, respectively. Overall, only 50.6% (N = 40,620) of all imputable markers were accurately imputed across ethnicities (Fig. 2A). Considering the HE included 158,878 non-monomorphic markers in this sample (among 243,783 total genotyped markers), only approximately one-quarter of variable HE content – and one-sixth of the total HE content – could be recapitulated from imputation via the HOE content. Note that we did not consider the small number of Y-chromosome (N = 180) and mtDNA markers (N = 245) available on the HE chip.

Imputation accuracy was also assessed separately for European Americans (N = 1476, Fig. 2B). We found a trend towards decreasing imputation accuracy with decreasing minor allele frequency. The proportion of markers which could be imputed accurately ($r^2 > 0.80$) was 65%. The small numbers of subjects in the other ancestry groups precluded statistical comparisons.

Finally, the total number of markers that could be imputed based on the HOE and HOEE, but not present on either platform, were considered. A large number of markers were successfully imputed at an acceptable quality (i.e., information threshold greater than 0.5) on both platforms (Supplemental Table 2). The total counts and overlap between HOE and HOEE were very similar. Only slightly more markers were imputed accurately using HOEE compared to HOE (22,961,598 and 22,898,511, respectively). Markers with rare variants (MAF < 0.01) accounted for roughly 54% of the approximately 23 million accurately imputed markers, while markers with common variants (MAF > 0.05) accounted for 30%. In general, there was high concordance of imputed genotypes between the HOE and HOEE (Supplemental Fig. S1). Approximately 17 million markers had $r^2 > 0.8$. Thus, the performance of the HOE and HOEE to impute markers not present on either platform was determined to be roughly equivalent.

3.3. Functional content for markers interrogated by the HE array

Of the 949,469 markers that passed genotyping QC (see Methods), the known or likely functional significance of 931,570 markers could be assessed using a suite of bioinformatics and computational procedures as described in (Torkamani et al., 2012) (see Methods). Of the 237,627 markers interrogated on the HE chip, there were 237,489 single-nucleotide variants (SNVs), 43 insertions, and 95 deletions. The classification of these markers into 9 functional groups is shown in Table 2 (left columns). Overall, 117,678 variants (49.5%) on the HE were predicted to be functional. When compared to the content on the more comprehensive HOEE array, we found that of the 122,668 HOEE functional variants, 117,678 (95.9%) were contributed by the HE. We also compared the contribution of functional content of the HE to the HOEE array after imputation (HOEEi; N = 22,961,598 markers amenable to imputation). We found that only approximately 0.7% of all variants capable of interrogation were likely to be functional (right columns of Table 2), suggesting that the HE chip is indeed substantially adding to the functional content available when using the HOE array, even after imputation. We note that some variants (N = 1143 or 0.12%) that were either interrogated on the HOEE chip or amenable to imputation were not amenable to functional prediction based on our computational procedures due to, for example, location inconsistencies in relevant databases.

3.4. Overall and functional variant frequencies

The majority of markers interrogated on the HE platform have very low minor allele frequencies. For example, 85% of markers exhibited minor allele frequency of 0.01 or less in our multi-ethnic cohort and similar trends were observed within each population. This observation has obvious implications on the utility of the HE in GWAS initiatives

Table 1
Descriptive statistics for the cohorts studied based on self-reported race and ethnicity.

Measure	Number of subjects	Males/females	Average age	#Poor genotype QC
Self-reported race:				
Black/African American	128	128/0	25.38	1
American Indian/Alaska	35	35/0	22.66	0
Asian	80	79/1	24.94	1
Pacific Island/Hawaiian	39	38/1	22.96	0
White	2104	2096/8	23.25	7
Multiple races	125	125/0	22.50	0
Unknown	46	46/0	23.19	0
Self-reported ethnicity:				
Non-Hispanic	1951	1946/5	23.42	8
Hispanic	601	596/5	23.18	1
Unknown	5	5/0	22.00	0
Total:	2557	2547/10	23.36	9

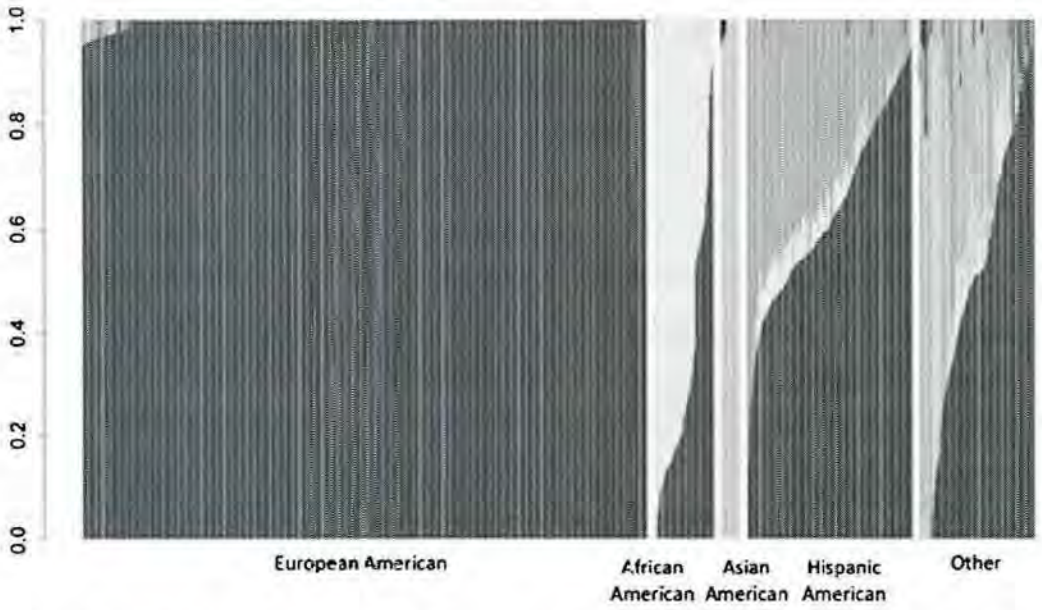

Fig. 1. Admixture proportion of individuals included in the study. Each individual is represented by a vertical bar divided into colored segments. The size of each colored segment reflects the proportion of admixture from one of six major continental populations (red – European; Yellow – African; green – Native American; turquoise – East Asian; blue – Oceanic; magenta – Central Asian). Individuals in each ancestral category are sorted by the degree of European admixture (i.e., size of red segments).

which focus on single marker tests. Assuming a small or moderate effect of variants on disease, most of the markers on the HE array will only provide sufficient power to detect associations between an allele and a disease using single marker tests if information on a very large number of case and control individuals is collected.

The mean (±s.d.) number of polymorphic markers per individual interrogated on the HE array was 15,746 (±215), and included 2454 (±59) functional markers, 14.3 (±6.4) private markers, and 7.9 (±3.8) functional and private markers. Similar numbers were seen in the European American subgroup (total: 15,523 ± 112; functional: 2420 ± 38; private: 10.1 ± 3.8, functional & private: 5.7 ± 2.6).

4. Discussion

As the genetics community learns about the limitations of contemporary approaches to discovering variants that influence phenotypic expression, newer approaches will undoubtedly emerge. It is quite clear that despite the spectacular and numerous successes in identifying associated variants via GWAS initiatives focusing on common variants and linkage disequilibrium phenomena, there is a large fraction of the genetic basis of most diseases and traits that has yet to be characterized. This could be due to one or more of the following factors: (1) rarity or relatively small effect sizes of the remaining variants contributing to those conditions; (2) forms of variation not hitherto explored in as comprehensive a manner as SNPs and small indels in GWAS initiatives (e.g., copy number of variants and large structural variations); (3) complicated gene x environment interactions; (4) epigenetic factors; and, (5) other phenomena (Frazer et al., 2009; Manolio et al., 2009; Schork et al., 2009).

The contribution of rare variants to phenotypic expression is getting more and more attention given the availability of cost-efficient sequencing technologies (Bansal et al., 2010; Bodmer and Bonilla, 2008; Frazer et al., 2009; Gibson, 2011; Malhotra and Sebat, 2012; Pasaniuc et al., 2012; Schork et al., 2009). However, sequencing technologies

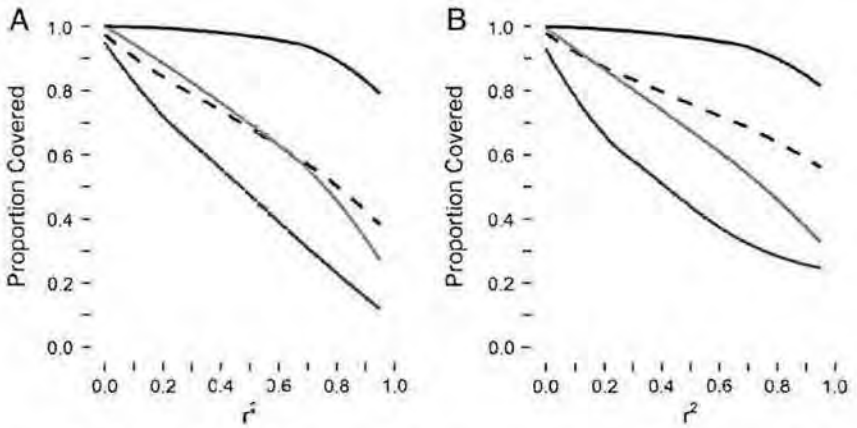

Fig. 2. The proportion of imputable markers (N = 80,205) exclusive to the HOEE (i.e. HE content) covered by imputation, based on the HOE and 1000 Genomes reference haplotypes across: A) all subjects (N = 2548); B) European Americans (N = 1475). Marker frequencies: blue – common (MAF > 0.05); green – moderately common (0.01 ≤ MAF ≤ 0.05); red – rare (MAF < 0.01); and black dashed – all.

Table 2
Functional content of the variants on the Human Exome array (HE) and the Human Omni ExpressExome plus imputable marker array (HOEEi) indicating the number of variants and rate in each of nine functional classes (see Methods).

Functional group	HE variants	Rate	HOEEi	Rate
Splicing change variants	372	0.030	625	0.015
Probably damaging nscSNPs	54,970	0.267	67,328	0.272
Possibly damaging nscSNPs	39,144	0.190	46,290	0.187
Protein motif damaging variants	23,304	0.292	27,283	0.293
TFBS disrupting variants	0	0.000	10	0.004
pre-miRNA disrupting variants	6	0.000	201	0.000
miRNA-BS disrupting variants	236	0.062	1931	0.055
ESE-BS disrupting variants	17,500	0.117	27,058	0.117
ESS-BS disrupting variants	6439	0.114	9869	0.116
Total likely functional variants	117,678	0.495	150,035	0.007

may still be cost-prohibitive for large-scale association studies. Therefore, the genetics research community has considered the use of genotyping platforms that can interrogate previously identified variants that are not easily captured via linkage disequilibrium on standard genotyping platforms *used in GWAS* initiatives. Choosing the markers to be used on such arrays is crucial, but a focus on coding variants (i.e., the exome) is a logical starting point (despite the fact that coding variants tend to be rare) since it has been shown that they are likely to be functional and have been implicated in a number of diseases and phenotypes (Botstein and Risch, 2003; Gorlov et al., 2011; Jordan et al., 2010; Sunyaev, 2012). However, designing a genotyping array that would complement existing genotyping platforms is not necessarily trivial. For example, imputation strategies are gaining sophistication making it possible to avoid the use of newer assays by computationally assigning variants to individuals based on linkage disequilibrium patterns in the genome and available data sets (Flannick et al., 2012; Marchini and Howie, 2010). Thus markers interrogated on newer platforms should optimally contain those not amenable to imputation. In addition, if markers are to be chosen for direct genotyping, then it makes sense to bias them towards those likely to include functional variants. Finally, many rare variants are likely to be population-specific, including those likely to be functional (Kidd et al., 2012; Torkamani et al., 2012), making the choice of which variants to include on a genotyping array complicated. For example, a researcher may not wish to invest in a genotyping platform if many of the markers being interrogated are not likely to be found in the populations of interest.

We explored these issues with a newly available genotyping array (the Illumina HE) designed to capture coding variants that are complementary to markers currently interrogated by other genotyping arrays. We find that as much as 49.5% of the markers interrogated by the array are likely to impact the function of genes. In addition, as only a small proportion of the HE content was amenable to imputation, we feel the addition of these markers provides an improvement over the previous GWAS array design — although it is possible that larger imputation reference panels may close this gap.

A limitation of our dataset is the unequal representation of different racial/ethnic groups with a relatively small number of Hispanics, African Americans, and subjects of other race, which precluded a detailed comparison of population-specific variants. In addition, our cohort was almost exclusively male, which effectively reduced the number of X chromosomes by half and did not allow for a comparison between genders. However, since analyses were based on the combined genomic content of the array, this should not impact our conclusions.

Obviously, the choice of a genotyping platform will have to be based on the goals of a study. For example, if a study requires the accommodation of *de novo*, very rare, or likely population-specific variants, then the use of an array designed to interrogate variants that have been previously identified is inappropriate. However, if the goal of a study is to efficiently expand the search for likely causative variants that are 'beneath the radar' of standard GWAS genotyping platforms, then genotyping arrays focusing on rare variants that are likely to be functional, such as coding variants, makes sense. The design of those arrays in terms of the variants they interrogate, however, is crucial for their success.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.01.069.

Conflict of interests

Dr. Schork is a Founder and hold stock in Cypher Genomics, a company employing one of the co-authors on this paper and draws salary from and holds stock in Human Longevity, Inc. a company employing one of the co-authors on this paper.

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RAPID PUBLICATION

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Blood-Based Gene-Expression Predictors of PTSD Risk and Resilience Among Deployed Marines: A Pilot Study

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Susceptibility to PTSD is determined by both genes and environment. Similarly, gene-expression levels in peripheral blood are influenced by both genes and environment, and expression levels of many genes show good correspondence between peripheral blood and brain. Therefore, our objectives were to test the following hypotheses: (1) pre-trauma expression levels of a gene subset (particularly immune-system genes) in peripheral blood would differ between trauma-exposed Marines who later developed PTSD and those who did not; (2) a predictive biomarker panel of the eventual emergence of PTSD among high-risk individuals could be developed based on gene expression in

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Stephen J. Glatt and Daniel S. Tylee contributed equally to this work.

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readily assessable peripheral blood cells; and (3) a predictive panel based on expression of individual exons would surpass the accuracy of a model based on expression of full-length gene transcripts. Gene-expression levels were assayed in peripheral blood samples from 50 U.S. Marines (25 eventual PTSD cases and 25 non-PTSD comparison subjects) prior to their deployment overseas to war-zones in Iraq or Afghanistan. The panel of biomarkers dysregulated in peripheral blood cells of eventual PTSD cases prior to deployment was significantly enriched for immune genes, achieved 70% prediction accuracy in an independent sample based on the expression of 23 full-length transcripts, and attained 80% accuracy in an independent sample based on the expression of one exon from each of five genes. If the observed profiles of pre-deployment mRNA-expression in eventual PTSD cases can be further refined and replicated, they could suggest avenues for early intervention and prevention among individuals at high risk for trauma exposure.

Key words: alternative splicing; mRNA; peripheral blood mononuclear cells; transcriptome; trauma

INTRODUCTION

Previous research on post-traumatic stress disorder (PTSD) has identified numerous factors that put individuals at greater risk of developing the disorder, such as family history, childhood or early adulthood experiences, personality and cognitive traits, and pre-existing mental disorders [Koenen et al., 2005; Kremen et al., 2007]; however, no easily assessed biological markers of PTSD have yet been validated. The biological factors associated with the risk for (and resilience to) PTSD are also poorly understood. Although susceptibility to PTSD appears to be moderately heritable, non-genetic factors (most prominently the type and extent of the precipitating trauma, and social support) and gene–environment interactions likely also contribute to each individual's overall susceptibility to the disorder [True et al., 1993; Stein et al., 2002; Kremen et al., 2012].

Given the less-than-absolute heritability of PTSD, pursuit of genetic markers alone (e.g., single nucleotide polymorphisms and copy-number variations) will leave much of the variance in vulnerability unexplained [Yehuda et al., 2011; Mehta and Binder, 2012]. Gene expression (i.e., mRNA) levels, which potentially reflect the effects of both heredity and environment, may be better indicators of the aberrant biology underlying PTSD, as well as its premorbid risk state. PTSD clearly is a brain disorder, but assaying gene-expression levels—either acutely or longitudinally—in the brains of living human subjects at risk for PTSD is impossible. Yet, as demonstrated by Sullivan et al. [2006] and, more recently, Rollins et al. [2010] and Kohane and Valtchinov [2012], peripheral blood expression levels of many genes are moderately correlated with the expression levels of those genes in other tissues, including *postmortem* brain, suggesting the possibility that peripheral blood gene expression can be harnessed to construct useful profiles of brain disorders [Woelk et al., 2011]. Indeed, we and others have capitalized on this proxy phenomenon to identify promising peripheral blood-based biomarkers for a number of neuropsychiatric disorders, including schizophrenia, bipolar disorder, and autism spectrum disorders [Glatt et al., 2005, 2009, 2011a,b, 2012; Tsuang et al., 2005; Lee et al., 2012].

In the context of PTSD, several prior studies identified differences in peripheral blood gene-expression levels between individuals with PTSD and similarly exposed comparison subjects without PTSD. First, Segman et al. [2005] described a longitudinal analysis of gene expression in peripheral blood mononuclear cells (PBMCs) from trauma survivors at the emergency room immediately after their trauma and again 4 months later when a diagnosis of PTSD could be definitively established. Predictably, this study found that the expression of many genes previously implicated in mediating the stress response (e.g., genes associated with hypothalamic–pituitary–adrenal [HPA] axis function) were significantly dysregulated in subjects with PTSD relative to those who fully recovered from their trauma. These changes in gene expression also showed a linear relationship with the severity of three different clusters of PTSD symptoms. In addition to changes in stress-response genes, the PBMCs from subjects with full persistent PTSD were marked by significant down-regulation of transcriptional activators, suggesting that subjects with PTSD may experience a global deficiency in the production of mRNAs (and, thus, proteins) of key genes at critical times. Subsequently, Zieker et al. [2007] replicated dysregulation of stress-response genes in whole blood from a sample of subjects with long-persistent PTSD resulting from the same environmental trigger (the Ramstein air show catastrophe, 1989). In addition, Zieker et al., extended earlier work by demonstrating changes in several immune-related genes among PTSD sufferers. In 2009, Yehuda et al. [2009] identified a profile of dysregulated genes in peripheral blood of survivors of the World Trade Center attacks that also was enriched with genes involved in HPA axis and immune cell functions. Most recently, Neylan et al. [2011] found global down-regulation of genes in CD14+ monocytes from male PTSD sufferers, but some evidence of increased activation of immune-system genes in female PTSD patients.

Consolidating this evidence with the results from epidemiologic, genomic, and neurobiological studies of the disorder [e.g., Uddin et al., 2010] led us to recently propose a theory of PTSD predicated on dysregulation of immune and inflammatory processes in general, and cellular immunity in particular [Baker et al., 2012b]. However, it was not clear from any of this work whether dysregulation of these processes occurs only in response to trauma exposure or if, in fact, gene-expression abnormalities in peripheral blood of individuals *exist "pre-trauma" and signal a heightened susceptibility* to developing the disorder once trauma is experienced. Recent work by van Zuiden et al. [2012] supports the assertion that pre-trauma disturbances in peripheral blood gene expression (at least in the realm of glucocorticoid signaling and regulation of cell-mediated immune and inflammatory processes) may predict post-trauma onset of PTSD and depressive symptoms.

We virtually never know about exposure to a traumatic event in advance, so the next best alternative in the pursuit of PTSD biomarkers has historically been studies of people who have recently experienced a trauma. But the critical limitation in such studies is that it is not possible to differentiate pre-existing risk factors from the consequences of trauma exposure or of develop-

ment of PTSD. In the context of this prior work, we report here the results of transcriptome-wide expression-profiling of peripheral blood samples from individuals at uniquely elevated risk of trauma exposure and development of PTSD: participants in the Marine Resiliency Study (MRS) *prior to their deployment* to active war zones in Iraq or Afghanistan, who were then followed longitudinally [Baker et al., 2012a]. The objectives of this pilot study were to evaluate the following hypotheses: (1) pre-trauma expression levels of some genes (particularly immune-system genes) in peripheral blood cells would differ between trauma-exposed Marines who later went on to develop PTSD and those who did not; (2) a readily assessable, predictive biomarker panel of the eventual emergence of PTSD among high-risk individuals could be developed based on gene expression levels in peripheral blood cells; and (3) a predictive panel based on the expression of individual exons would surpass the accuracy of a model based on the expression of full-length transcripts of genes. We interpret the results of these analyses in two contexts: (1) as a means of identifying biological functions, processes, pathways, and protein domains whose genomic dysregulation may indicate or influence susceptibility to the disorder; and (2) the construction of predictive or prognostic classifiers that might ultimately find use in assessing individual risk for PTSD and implementing preventive strategies in such populations.

METHODS

Ascertainment and Clinical Characterization of Subjects

The MRS is a prospective cohort study of factors predictive of PTSD among approximately 2,600 Marines in four battalions deployed to Iraq or Afghanistan. The research team conducted structured clinical interviews on Marine bases and collected blood samples and data at four time points: pre-deployment, and ~1-week, ~3-months, and ~6-months after returning from deployment (i.e., post-deployment). Measures collected, including those used in this study, have been described in detail previously [Baker et al., 2012a].

The principal exclusion criteria for both affected cases and unaffected comparison subjects for the present analyses were: (1) a pre-deployment PTSD Checklist (PCL) score >44; and/or (2) a pre-deployment diagnosis of PTSD based on the Clinician-Administered PTSD Scale (CAPS). In other words, no included subjects met either clinician- or self-rated thresholds for a diagnosis of PTSD at pre-deployment. Cases were identified as those subjects who were issued a CAPS-based PTSD diagnosis at ~3- and/or ~6-months post-deployment. Unaffected comparison subjects were identified as those subjects who, at no time, attained a PCL score >44 and who were not issued a CAPS-based PTSD diagnosis at any post-deployment interview. Among subjects who were included in the full MRS sample and assigned to case or comparison groups based on these criteria, we then selected for analysis 25 male PTSD cases and 25 male comparison subjects based on similar demographics, pre-deployment clinical characteristics, deployment history, and levels of exposure to putative traumas as determined from the Combat and Post-Battle Experiences subscales of the Deployment Risk and Resilience Inventory (DRRI). After performing quality-control checks on the microarray data (described below), two subjects (one case and one comparison subject) were removed from analyses. The demographic, clinical, and combat-experiential characteristics of the remaining 24 case and 24 comparison subjects are shown in Table I. The two groups were comparable on all demographic and combat-experiential variables. Within both the case and comparison groups, 50% of the subjects had been deployed previously on at least one occasion, and while some subjects in each group had been previously deployed multiple times (up to three

TABLE I. Demographic, Clinical, and Experiential Characteristics of Eventual PTSD Cases and Non-PTSD Comparison Subjects

	Eventual PTSD cases	Comparison subjects	*P*-value
Sample size: *n*	24	24	
Age:	21.9 ± 3.2	21.5 ± 3.2	0.653
Previously deployed: *n* (%)	12 (50.0)	12 (50.0)	1.000
Ancestry: Caucasian *n* (%)	17 (70.8)	18 (75.0)	0.853
Cohort *n* (%)			
1	2 (8.3)	5 (20.8)	0.471
2	8 (33.3)	7 (29.2)	
3	14 (58.3)	12 (50.0)	
DRRI combat experiences	18.9 ± 13.1	20.2 ± 14.9	0.754
DRRI Post-battle experiences	7.3 ± 4.6	8.7 ± 4.0	0.281
CAPS pre-deployment	22.6 ± 12.0	15.4 ± 9.7	0.027
CAPS 3-months post-deployment	67.2 ± 21.8	40.0 ± 29.4	0.013
PCL Pre-Deployment	24.6 ± 6.4	23.2 ± 3.4	0.346
PCL 1-Week Post-Deployment	42.7 ± 17.6	23.0 ± 4.9	<0.001
PCL 3-months post-deployment	49.3 ± 12.5	21.2 ± ± 4.6	<0.001
PCL 6-months post-deployment	40.6 ± 13.8	20.1 ± 2.6	<0.001

Notes: (1) Demographic characteristics of each sample are reported as mean ± SD unless otherwise noted. (2) Sample means and proportions were compared using independent samples *t*-tests and chi-square tests, respectively.

times), there was no difference between the two groups in the proportion of multiply deployed individuals or in the average number of deployments. Although no subject met diagnostic threshold for PTSD at pre-deployment as determined by either clinician ratings on the CAPS or self-ratings on the PCL, the eventual PTSD cases did have significantly higher clinician ratings on the CAPS at pre-deployment, whereas no significant difference in pre-deployment self-ratings on the PCL were observed. As expected, the eventual PTSD cases also had significantly higher clinician- and self-rated symptoms of PTSD at all post-deployment evaluations.

mRNA Sample Acquisition, Stabilization, Isolation, and Storage

Close collaboration with the Marine Corps and the Navy, which provides health support for the Marine Corps, enabled comprehensive on-site data collection. The clinical interview and sample blood draw (10 ml) were both collected within 4 hr of each other on the same day. Each blood sample was collected into an EDTA-coated collection tube and immediately transferred to an RNase-free laboratory, where all subsequent procedures took place. The blood sample was passed over a LeukoLOCK filter, which was flushed with PBS and then fully saturated with RNAlater [Gonzales et al., 2005]. Each LeukoLOCK filter, containing bound, isolated, stabilized, and purified white blood cells, was sealed and stored in a sterile box at −20°C. Once mRNA samples were acquired from all subjects, the entire batch of samples was processed to isolate mRNA. Eluted mRNA samples were stored at −80°C until transferred to the SUNY MicroArray Core (SUNYMAC, Syracuse, NY) Facility at SUNY Upstate Medical University for quality assurance and microarray hybridization. LeukoLOCK filters, RNAlater, and TRI reagent were obtained from Applied Biosystems, Inc. (Foster City, CA), while all other reagents and supplies were obtained from VWR International, LLC (West Chester, PA) unless otherwise specified.

mRNA Quantitation, Quality Control, and Hybridization

The concentration of mRNA in each DNA-free sample was quantified by the absorption of ultraviolet light at two wavelengths (260 and 280 nm), which was measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific; Wilmington, DE). The quantity of mRNA in each of the 50 samples far exceeded the minimally sufficient amount required for microarray hybridization. The purity of each mRNA sample was estimated by the 260:280 nm absorbance ratio, with an acceptable range designated a priori as 1.7–2.1. The quality of each mRNA sample was quantified by the RNA Integrity Number (RIN) [Schroeder et al., 2006], which was determined on an RNA 6000 Labchip Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). According to convention [Schroeder et al., 2006], a RIN of 6.0 or greater was deemed to be indicative of acceptable quality, and no samples were removed based on this criterion. Two batches of 25 samples each (balanced with PTSD cases and controls) were then assayed on GeneChip Human Exon 1.0 ST Arrays (Affymetrix, Inc., Santa Clara, CA) per the "Whole Transcript Sense Target Labeling Assay" protocol [Affymetrix, 2006] using 1 μg of total RNA from each sample.

Microarray Data Import, Normalization, Transformation, Summarization, and Quality Control

Partek Genomics Suite software, version 6.6 © 2012 (Partek Incorporated, St. Louis, MO), was utilized for all analytic procedures performed on microarray scan data. Interrogating probes were imported, and corrections for background signal were applied using the robust multi-array average (RMA) method [Irizarry et al., 2003], with additional corrections applied for the GC-content of probes. The set of GeneChips was standardized using quantile normalization and expression levels of each probe underwent log-2 transformation to yield distributions of data that more closely approximated normality. As most genes were measured by multiple probe sets (typically one probe set per exon, but sometimes more), summarization of probes took place at two levels: first, probes tagging the same exon were summarized by median polish to arrive at one expression value per exon; second, exons tagging the same gene were summarized by median polish to arrive at one expression value per gene. All probesets were expressed with a signal:noise ratio ≥ 3; thus, no probesets were excluded from analyses of differential expression. A total of 257,106 probesets were analyzed, mapping to 20,224 whole transcripts and 209,826 exons.

Unsupervised clustering of subjects revealed no evidence of batch effects based on scan date. Principal components analysis (PCA) of the 50 pre-deployment data points identified two outliers (one case and one comparison subject) whose component values were beyond four standard deviations (SD) in each of the first three dimensions of the PCA plot, suggesting that the fundamental gene-expression pattern measured in these subjects (as evidenced by correlations among expression levels of probes) was inconsistent with that of the majority of other subjects. Both outlier samples exhibited high levels of average deviation among redundant probes located within a given chip, as well as high levels of average deviation in comparison with the median expression levels across all chips, suggesting either physical defects or hybridization problems with these chips. Removal of these two samples resulted in all 48 remaining subjects' data being well within the four-SD ellipsoid on each of the first three PCA dimensions.

Microarray Data Analyses

We performed four independent sets of analyses on the microarray data, as described below.

Identification of differentially expressed genes and their associated biological terms. We utilized analyses of covariance (ANCOVAs) to determine which full-length genetic transcripts were differentially expressed at pre-deployment in peripheral blood cells between PTSD cases and comparison subjects. We performed ANCOVAs of each gene's expression level as a function of PTSD status (case or control), deployment cohort (three levels corresponding to three platoons deployed at different times), age

(continuously measured in years), ancestry (dichotomized as Caucasian or not, as most subjects were Caucasian), and prior deployment status (first or subsequent deployment). Prior deployment accounted for less global variation in the expression dataset than did error, and prior deployment rates did not differ significantly between cases and comparison subjects, so it was removed from the model and subsequent analyses to preserve degrees of freedom.

To generate a relatively large candidate-gene list for functional profiling and construction of classifiers, we set the uncorrected type-I-error rate for diagnosis in these analyses at 0.01. We then reduced the dimensionality of the resulting list of candidate biomarkers through analysis of annotation-enrichment using the DAVID algorithm [Dennis et al., 2003] to determine if the gene list disproportionately represented any biological "terms." Specifically, we evaluated whether the list was enriched with genes that aggregated in the same functional categories, represented similar ontologies, participated in the same biological pathways, or exhibited common protein domains.The evaluated terms included: (1) ontologies from Gene Ontology Consortium (GOC) [Ashburner et al., 2000] and Clusters of Orthologous Groups (COG) [Tatusov et al., 2000]; (2) keywords from the Protein Information Resource (PIR) [Wu et al., 2003]; (3) features from the Universal Protein Resource (UniProt) [Apweiler et al., 2004]; (4) biological pathways from BioCarta and the Kyoto Encyclopedia of Genes and Genomes (KEGG) [Kanehisa and Goto, 2000]; and (5) protein domains from PIR, the Integrative Protein Signature database (InterPro) [Hunter et al., 2009], the Simple Modular Architecture Research Tool (SMART) [Schultz et al., 1998], and the University of California at Santa Cruz's Transcription Factor Binding Site (TFBS) database. Bonferroni-correction was applied to the *P*-values obtained in the enrichment analyses of these annotation terms, and we only considered significant those tests that exceeded a threshold of $P = (0.05/\text{the number of terms evaluated in a particular category})$.

Discovery and replication of gene-based diagnostic predictors. We utilized a machine-learning technique (support vector machine, SVM) to construct, evaluate, optimize, and cross-validate classification algorithms predicting eventual PTSD status based on gene-expression levels at pre-deployment for a subset of our full sample. To accomplish this, we generated a large list of differentially expressed candidate genes (nominal $P < 0.01$) in a subset of the sample (19 cases and 19 comparison subjects) using ANCOVA and the same panel of factors and covariates described above. The probes on this list were then supplied as potential predictors in an SVM, as various model parameters and predictor combinations were evaluated to identify the model with the highest accuracy in identifying cases and comparison subjects based solely on the expression levels of a minimal gene set identified by shrinking centroids after two-level nested (i.e., two-level) 10-fold cross-validation. The top-performing model was then deployed on a fully independent test sample (five cases and five comparison subjects) to determine its generalizability in accurately predicting case status based on gene-expression levels (the 10 subjects used for model validation were not significantly different from those in the training set in terms of demographic, gene-expression QC, experiential, or clinical factors; data not shown).

Identification of differentially expressed exons and their associated biological terms. We examined exon-expression levels utilizing ANCOVAs to identify putative alternative splicing differences between individuals who would go on to develop PTSD and those who would not. The same factors evaluated in gene-based analyses (PTSD status, cohort, age, and ancestry) were assessed for their main effects and their interaction with exon ID as predictors of exon-expression levels, c.f. [Glatt et al., 2009]; however, due to the stronger effects of diagnosis on exon-specific expression observed relative to the earlier gene-based analyses, we restricted the candidate-gene list to transcripts with $P < 0.0001$ for the interaction of diagnosis and exon ID. This yielded a gene list still sufficiently large for the construction of classifiers (see below) and enrichment analyses, which we again performed using the DAVID algorithm. Enrichments were evaluated against a Bonferroni-corrected *P*-value accounting for the number of terms evaluated.

Discovery and replication of exon-based diagnostic predictors. As outlined above for full-length transcripts under Methods Section, we used SVMs to construct, evaluate, optimize, and cross-validate classification algorithms predicting eventual PTSD status based on exon-expression levels at pre-deployment for the same subset of our full sample. We first generated a large candidate list of putatively alternatively spliced genes (nominal $P < 0.0001$ for the interaction of PTSD status and exon ID) in a subset of the sample (19 cases and 19 comparison subjects) using ANCOVA and the same panel of factors, covariates, and interaction terms described above. For each gene on the list, the most significantly dysregulated exon was identified and supplied as a potential predictor in the SVM classifiers. Various model parameters and predictor combinations then were evaluated to identify the model with the highest accuracy in identifying cases and comparison subjects based solely on the expression levels of a minimal exon set identified by shrinking centroids after two-level nested 10-fold cross-validation. The top-performing model was then deployed on the fully independent test sample (five cases and five comparison subjects) to determine its generalizability in accurately predicting case status based on exon-expression levels.

RESULTS

Identification of Differentially Expressed Genes and Their Associated Biological Terms

No gene's expression level was related to future PTSD status at a Bonferroni-corrected level of significance, which is not surprising given the relatively small sample size and large number of transcripts tested. We did, however, identify 67 probes dysregulated with a nominally significant $P < 0.01$ in Marines who were later diagnosed with PTSD (Table II). Thirty-nine of these 67 probes were down-regulated, whereas 28 were up-regulated. While the direction of this pattern is consistent with prior work identifying transcriptional down-regulation in PTSD [Segman et al., 2005; Neylan et al., 2011], the ratio of down-regulated to up-regulated probes was not significantly different from chance expectation (one-tailed sign-test, $P = 0.11$). Log 2 fold-change (FC) of these probes in eventual PTSD cases ranged from 1.8-fold down-regulation to 2.1-fold up-regulation. Annotations significantly enriched in the list of 59 genes tagged by the 67 dysregulated probes—after Bonferroni correction for the number of terms

TABLE II. Genes Significantly Dysregulated ($P < 0.01$) in Peripheral Blood Mononuclear Cells from the Full Sample of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers

			Diagnostic group main effect		
Transcript cluster ID	Gene symbol	Gene product	Fold-change in cases	*F*	*P*-value
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	2.14	8.9	4.6E−03
7902541	*IFI44L*	Interferon-induced protein 44-like	1.77	7.8	7.9E−03
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3, 100 kDa	1.72	7.5	8.8E−03
7971296	*EPSTI1*	Epithelial stromal interaction 1 (breast)	1.68	11.7	1.4E−03
8050102	*CMPK2*	Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	1.54	9.2	4.1E−03
8071155	*USP18*	Ubiquitin specific peptidase 18	1.49	7.4	9.5E−03
7921434	*AIM2*	Absent in melanoma 2	1.46	8.8	4.9E−03
8046124	*DHRS9*	Dehydrogenase	1.44	8.9	4.7E−03
7958884	*OAS1*	2′,5′-oligoadenylate synthetase 1, 40	1.39	10.6	2.2E−03
7958913	*OAS2*	2′,5′-oligoadenylate synthetase 2, 69	1.38	8.5	5.7E−03
8004184	*XAF1*	XIAP associated factor 1	1.29	8.7	5.2E−03
7976443	*IFI27*	Interferon, alpha-inducible protein 27	1.26	11.6	1.4E−03
7953924	*CLEC9A*	C-type lectin domain family 9, member A	1.22	7.4	9.5E−03
8121532	*WISP3*	WNT1 inducible signaling pathway protein 3	1.22	8.6	5.3E−03
8107094	*ENST00000442824*	Cdna:pseudogene chromosome: GRCh37:5:97549106:97549825:	1.20	7.9	7.5E−03
8043375	*TRNK*	Mitochondrially encoded tRNA lysine	1.19	7.4	9.3E−03
8060294	*PDCD1*	Programmed cell death 1	1.18	8.2	6.5E−03
8127234	*DST*	Dystonin	1.15	9.0	4.4E−03
8018315	*SUMO2*	SMT3 suppressor of mif two 3 homolog 2 (*S. cerevisiae*)	1.14	9.9	2.9E−03
8060997	*SPTLC3*	Serine palmitoyltransferase, long chain base subunit 3	1.13	8.0	7.2E−03
8118345	*CFB*	Complement factor B	1.12	8.1	6.8E−03
8162884	*ALDOB*	Aldolase B, fructose-bisphosphate	1.11	10.5	2.3E−03
8061847	*C20orf70*	Chromosome 20 open reading frame 70	1.11	8.5	5.6E−03
8178115	*CFB*	Complement factor B	1.11	9.9	3.0E−03
7963386	*KRT82*	Keratin 82	1.10	9.2	4.1E−03
7990391	*CYP1A1*	Cytochrome P450, family 1, subfamily A, polypeptide 1	1.09	10.0	2.9E−03
8069503	*LOC441956*	Similar to cDNA sequence BC021523	1.09	19.0	8.0E−05
8139721	*ENST00000462919*	ncrna_pseudogene:miRNA_pseudogene chromosome: GRCh37:7:55713765:55713874	1.07	7.5	9.0E−03
7993146	*ENST00000475032*	ncrna_pseudogene:scRNA_pseudogene chromosome: GRCh37:16:8777112:8777408	−1.05	8.2	6.4E−03
8027824	*MAG*	Myelin associated glycoprotein	−1.08	7.6	8.5E−03
8142685	*TMEM229A*	Transmembrane protein 229A	−1.08	8.8	4.9E−03
8065252	*ENST00000432334*	cdna:known chromosome: GRCh37:20:19738352:19780320	−1.08	8.6	5.4E−03
8030002	*ZNF114*	Zinc finger protein 114	−1.09	9.6	3.4E−03
8118455	*C4A*	Complement component 4A (Rodgers blood group)	−1.09	9.1	4.3E−03
7945498	*SCT*	Secretin	−1.09	11.6	1.4E−03
8179399	*C4A*	Complement component 4A (Rodgers blood group)	−1.09	7.7	8.3E−03
8100523	*SPINK2*	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	−1.10	8.4	5.8E−03
8152812	*FAM84B*	Family with sequence similarity 84, member B	−1.10	10.3	2.6E−03
8024816	*FSD1*	Fibronectin type III and SPRY domain containing 1	−1.11	9.3	4.0E−03
8137962	*LOC100129484*	Hypothetical LOC100129484	−1.11	9.2	4.1E−03
8060339	*NRSN2*	Neurensin 2	−1.12	9.6	3.5E−03
7920264	*S100A5*	S100 calcium binding protein A5	−1.13	7.9	7.3E−03
8018646	*FOXJ1*	Forkhead box J1	−1.14	10.5	2.3E−03
8051061	*UCN*	Urocortin	−1.14	9.4	3.8E−03
8129095	*ENST00000435100*	Cdna:pseudogene chromosome: GRCh37:6:116579656:116580278	−1.15	7.6	8.7E−03
8122699	*RPS18P9*	Ribosomal protein S18 pseudogene 9	−1.15	8.9	4.6E−03

TABLE II. (*Continued*)

Transcript cluster ID	Gene symbol	Gene product	Diagnostic group main effect: Fold-change in cases	*F*	*P*-value
8012891	*ENST00000412454*	Cdna:pseudogene chromosome: GRCh37:17:14608393:14608851	−1.15	8.4	5.8E−03
8071368	*TMEM191A*	Transmembrane protein 191A	−1.15	8.9	4.7E−03
8127526	*RPL39*	Ribosomal protein L39	−1.15	8.0	7.2E−03
7985192	*AGPHD1*	Aminoglycoside phosphotransferase domain containing 1	−1.16	7.6	8.7E−03
8072584	*ENST00000042361*	Cdna:pseudogene chromosome: GRCh37:22:32435477:32435883	−1.16	9.4	3.7E−03
7992678	*LOC652276*	Hypothetical LOC652276	−1.16	7.4	9.5E−03
8118974	*RPL10A*	Ribosomal protein L10a	−1.17	9.3	3.9E−03
8147112	*CA13*	Carbonic anhydrase XIII	−1.17	8.1	6.7E−03
8063410	*PARD6B*	par-6 partitioning defective 6 homolog beta (C. elegans)	−1.17	8.5	5.5E−03
8148923	*LRRC14*	Leucine rich repeat containing 14	−1.18	7.8	7.8E−03
7953032	*LRTM2*	Leucine-rich repeats and transmembrane domains 2	−1.19	7.5	9.0E−03
8076260	*SLC25A17*	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34 kDa), member 17	−1.19	8.3	6.1E−03
7982271	*GOLGA8IP*	Golgin A8 family, member I (pseudogene)	−1.20	8.6	5.5E−03
7991742	*MPG*	*N*-methylpurine-DNA glycosylase	−1.20	9.2	4.1E−03
7905691	*RPS27*	Ribosomal protein S27	−1.20	7.7	8.0E−03
7950753	*CCDC90B*	Coiled-coil domain containing 90B	−1.24	10.2	2.6E−03
8103622	*CBR4*	Carbonyl reductase 4	−1.27	8.1	6.6E−03
8107520	*TNFAIP8*	Tumor necrosis factor, alpha-induced protein 8	−1.30	8.1	6.7E−03
7909601	*SNORA16B*	Small nucleolar RNA, H	−1.32	8.0	7.0E−03
8154962	*DNAJB5*	DnaJ (Hsp40) homolog, subfamily B, member 5	−1.35	10.4	2.4E−03
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	−1.83	10.2	2.7E−03

*Rows are sorted by decreasing fold change in eventual PTSD cases relative to non-PTSD comparison subjects.

evaluated—included most prominently immune-related processes and protein domains involved in the response to viral infection (Table III), most of which were up-regulated in future PTSD cases. Exploratory pathway analysis of the differentially expressed genes in Table II using the Reactome database [Matthews et al., 2009] revealed that a subset of genes involved in type-1 interferon signaling represented the only significantly enriched pathway within our dataset. Six of the 59 genes were differentially expressed (*IFI27*, *OAS1*, *OAS2*, *OAS3*, *XAF1*, and *USP18*), with all probes up-regulated in future PTSD cases.

Discovery and Replication of a Gene-Based Diagnostic Predictor

To construct a gene-based classifier and assess its generalizability, we first derived a list of potential classifier transcripts as those probes with a difference in expression between PTSD case and comparison subjects attaining $P < 0.01$ in the training sample of 19 cases and 19 comparison subjects while controlling for the same factors and covariates as in analysis 1. This analysis and filtering left 61 probes (Table IV) that were then used to build and optimize SVM classifiers. The optimal SVM (identified through two-level nested 10-fold cross-validation with shrinking centroids, cost = 401, tolerance = 0.001, kernel = radial basis function, and gamma = 0.001) comprised 23 of the 61 starting probes (Table IV, probes in bold font) and attained 85% accuracy in classifying those individuals in the training sample who would or would not go on to develop PTSD. We then tested the identical 23-gene SVM (with the same parameters, but with no shrinkage or cross-validation) in the remaining independent test cohort (five cases and five comparison subjects), where it yielded a diminished but still reasonable 70% accuracy. Among cases, three of five were correctly classified, while four of five comparison subjects were classified correctly. These values correspond to a sensitivity, specificity, positive predictive value, and negative predictive value in the test sample of 60%, 80%, 75%, and 67%, respectively.

Identification of Differentially Expressed Exons and Their Associated Biological Terms

The interaction of diagnosis and exon ID identified putative isoform-expression differences ($P < 0.0001$) in 13 genes, seven of which attained Bonferroni-corrected significance (Table V). An example of between-group differences in exon expression for one of these five genes (*SUV420H1*) is illustrated in Figure 1, where the future PTSD cases have significantly lower levels of expression of a

TABLE III. Annotations Enriched at Bonferroni-Corrected Significant Levels Among Genes Dysregulated ($P < 0.01$) in Peripheral Blood Mononuclear Cells From the Full Sample of Eventual PTSD Cases at Pre-Deployment*

Category	Term	Count (%)	Fold enrichment	*P*-value	Bonferroni corrected *P*	Dysregulated genes (direction of dysregulation in eventual PTSD cases)
GOTERM_BP_FAT	GO:0006955 ~ immune response	10 (2.2)	5.6	3.7E−05	1.8E−02	*OAS1*(↑), *OAS2*(↑), *AIM2*(↑), *PDCD1*(↑)
INTERPRO	IPR006117:2′,5′-oligoadenylate synthetase, conserved site	3 (0.7)	277.7	4.1E−05	5.7E−03	*OAS3*(↑), *OAS1*(↑), *OAS2*(↑)
INTERPRO	IPR018952:2′,5′-oligoadenylate synthetase 1, domain 2/C-terminal	3 (0.7)	277.7	4.1E−05	5.7E−03	*OAS3*(↑), *OAS1*(↑), *OAS2*(↑)
INTERPRO	IPR006116:2′,5′-oligoadenylate synthetase, ubiquitin-like region	3 (0.7)	222.1	6.8E−05	9.5E−03	*OAS3*(↑), *OAS1*(↑), *OAS2*(↑)

*Rows are sorted by increasing *P*-value for the enrichment of annotations.

TABLE IV. Genes Significantly Dysregulated ($P < 0.01$) in Peripheral Blood Mononuclear Cells From a Subset of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers*

			Diagnostic group main effect		
Transcript cluster ID[a]	Gene symbol	Gene product	Fold-change in cases	*F*	*P*-value
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	2.33	9.3	4.6E−03
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3, 100 kDa	2.01	11.0	2.3E−03
7902541	*IFI44L*	Interferon-induced protein 44-like	1.99	11.0	2.3E−03
8064716	*SIGLEC1*	Sialic acid binding Ig-like lectin 1, sialoadhesin	1.48	10.4	2.9E−03
7958913	*OAS2*	2′,5′-Oligoadenylate synthetase 2, 69	1.45	8.0	8.2E−03
8165682	*TRNS2*	Mitochondrially encoded tRNA serine 2	1.38	8.2	7.5E−03
8102127	*TACR3*	Tachykinin receptor 3	1.35	7.6	9.6E−03
7971191	*SUGT1P3*	Suppressor of G2 allele of SKP1 (S. cerevisiae) pseudogene 3	1.27	8.4	6.7E−03
8043375	*TRNK*	Mitochondrially encoded tRNA lysine	1.25	8.4	6.8E−03
8165684	*TRNL2*	Mitochondrially encoded tRNAleucine 2	1.25	8.6	6.2E−03
8165667	*TRNK*	Mitochondrially encoded tRNA lysine	1.25	8.1	7.5E−03
7896752	*TRNK*	Mitochondrially encoded tRNA lysine	1.25	8.1	7.5E−03
8055594	*ENSEMBL*	ncrna_pseudogene:Mt_tRNA_pseudogene chromosome: GRCh37:2:1	1.23	8.2	7.3E−03
7903203	*SNX7*	sorting nexin 7	1.21	9.4	4.4E−03
7938561	*ENST00000487144*	ncrna_pseudogene:rRNA_pseudogene chromosome: GRCh37:11:132	1.16	10.8	2.4E−03
8087433	*NICN1*	Nicolin 1	1.16	7.9	8.3E−03
8060997	*SPTLC3*	Serine palmitoyltransferase, long chain base subunit 3	1.15	9.0	5.1E−03
8031680	*ENST00000492903*	ncrna_pseudogene:Mt_tRNA_pseudogene chromosome: GRCh37:19:	1.13	8.3	7.1E−03
7953697	*GENSCAN00000020682*	cdna:Genscan chromosome: GRCh37:12:8090472:8168935:1	1.07	8.1	7.5E−03
8141423	*MIR106B*	microRNA 106b	−1.05	9.3	4.5E−03
8091099	*ENST00000450495*	cdna:known chromosome: GRCh37:3:141583849:141584121:-1 gen	−1.06	8.7	6.0E−03
8146643	*MIR124-2*	microRNA 124-2	−1.07	8.3	7.0E−03
8027824	*MAG*	Myelin associated glycoprotein	−1.08	8.2	7.3E−03
7911941	*CHD5*	Chromodomain helicase DNA binding protein 5	−1.08	8.3	6.9E−03
7955211	*DNAJC22*	DnaJ (Hsp40) homolog, subfamily C, member 22	−1.08	9.4	4.4E−03

TABLE IV. (*Continued*)

Transcript cluster ID[a]	Gene symbol	Gene product	Diagnostic group main effect: Fold-change in cases	*F*	*P*-value
8055314	*LYPD1*	LY6	−1.09	7.6	9.7E−03
8065252	*BC004382*	*Homo sapiens*, clone IMAGE:3640982, mRNA, partial cds	−1.10	7.7	9.0E−03
8100523	*SPINK2*	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibi	−1.10	11.2	2.1E−03
8030002	*ZNF114*	Zinc finger protein 114	−1.11	15.2	4.6E−04
8060339	*NRSN2*	Neurensin 2	−1.11	13.1	9.9E−04
8152812	*FAM84B*	Family with sequence similarity 84, member B	−1.11	7.9	8.3E−03
8112072	*CCNO*	Cyclin O	−1.11	7.7	9.3E−03
7945498	*SCT*	Secretin	−1.12	14.8	5.3E−04
8126450	*RPL24*	Ribosomal protein L24	−1.13	7.5	9.8E−03
8084478	*FAM131A*	Family with sequence similarity 131, member A	−1.13	8.1	7.7E−03
8042532	*VAX2*	Ventral anterior homeobox 2	−1.13	17.5	2.1E−04
8151281	*TRAM1*	Translocation associated membrane protein 1	−1.13	8.4	6.7E−03
8019687	*ANAPC11*	Anaphase promoting complex subunit 11	−1.14	9.4	4.4E−03
8074869	*RTDR1*	Rhabdoid tumor deletion region gene 1	−1.14	8.1	7.8E−03
7928107	*H2AFY2*	H2A histone family, member Y2	−1.14	8.0	7.9E−03
8024816	*FSD1*	Fibronectin type III and SPRY domain containing 1	−1.15	12.2	1.4E−03
8038048	*CCDC114*	Coiled-coil domain containing 114	−1.15	9.3	4.5E−03
8084982	*LOC152217*	Hypothetical LOC152217	−1.15	7.6	9.7E−03
8127526	*RPL39*	Ribosomal protein L39	−1.17	8.8	5.6E−03
8154563	*ACER2*	Alkaline ceramidase 2	−1.18	9.9	3.5E−03
7985192	*AGPHD1*	Aminoglycoside phosphotransferase domain containing 1	−1.18	7.9	8.5E−03
8178090	*C6orf48*	Chromosome 6 open reading frame 48	−1.18	7.9	8.5E−03
8179326	*C6orf48*	Chromosome 6 open reading frame 48	−1.18	7.9	8.5E−03
8018646	*FOXJ1*	Forkhead box J1	−1.18	9.8	3.7E−03
8072584	*ENST00000423610*	cdna:pseudogene chromosome: GRCh37:22:32435477:32435883:1	−1.19	8.7	5.9E−03
8022170	*RPL6*	Ribosomal protein L6	−1.20	9.0	5.2E−03
7932964	*C1D*	C1D nuclear receptor corepressor	−1.21	8.9	5.4E−03
8085852	*NGLY1*	*N*-glycanase 1	−1.22	9.3	4.6E−03
8160308	*RPS6*	Ribosomal protein S6	−1.22	9.8	3.7E−03
8038993	*ZNF28*	Zinc finger protein 28	−1.25	8.4	6.8E−03
8107520	*TNFAIP8*	Tumor necrosis factor, alpha-induced protein 8	−1.29	7.9	8.5E−03
7911359	*NOC2L*	Nucleolar complex associated 2 homolog (S. cerevisiae)	−1.29	7.8	8.9E−03
8119357	*DAAM2*	Dishevelled associated activator of morphogenesis 2	−1.30	9.8	3.7E−03
8090256	*SNX4*	Sorting nexin 4	−1.37	8.8	5.6E−03
8155359	*CNTNAP3*	Contactin associated protein-like 3	−1.42	7.7	9.1E−03
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	−1.95	9.2	4.8E−03

[a]Rows are sorted by decreasing fold-change in eventual PTSD cases relative to non-PTSD comparison subjects.
[b]Transcripts in bold comprised the optimal 23-probe SVM classifier of eventual PTSD status identified by training and testing in independent samples.

single probe in the 3′ (left) end of the gene suggesting lower expression of the *b* isoform (one of the gene's 12 known isoforms) among future PTSD cases. The list of 13 genes was analyzed by the DAVID algorithm, but no annotations were found to be significantly enriched after Bonferroni correction for the number of terms evaluated; this is not surprising based on the small size of this gene list, which did not afford much opportunity for enrichment to be detected.

Discovery and Replication of an Exon-Based Diagnostic Predictor

To construct an exon-based classifier and assess its generalizability we first identified potentially differentially spliced exons within our training sample of 19 cases and 19 comparison subjects based on the diagnosis × exon ID interaction term, using a nominal threshold of $P < 0.0001$, while controlling for the same factors and covariates

TABLE V. Exons Significantly Dysregulated in Peripheral Blood Mononuclear Cells From the Full Sample of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers*

Transcript cluster ID	Gene symbol	Gene product	Accession number	*F*	*P*	Adjusted *P*	q	Probesets (n)	Dysregulated probeset IDs[a]
7954810	*LRRK2*	Leucine-rich repeat kinase 2	NM_198578	3.22	2.0E−13	4.1E−09	4.1E−09	53	7954813, 7954814, 7954818, **7954820**, 7954832, 7954833, 7954845, 7954854, 7954856, 7954863
8068740	*UMODL1*	Uromodulin-like 1	NM_001004416	5.28	3.4E−12	3.4E−08	6.9E−08	20	**8068745**, 8068747
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	NM_080657	7.94	8.5E−10	5.7E−06	1.7E−05	9	8040082, 8040083, 8040084, **8040085**, 8040086, 8040087, 8040088
7949931	*SUV420H1*	Suppressor of variegation 4-20 homolog 1 (Drosophila)	NM_017635	5.06	1.7E−08	8.6E−05	3.4E−04	14	**7949933**
8136662	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	NM_004668	2.38	1.0E−06	4.1E−03	0.02	46	—
8163535	*AMBP*	Alpha-1-microglobulin	NM_001633	4.18	6.6E−06	2.2E−02	0.13	12	8163538, **8163541**, 8163547
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	NM_000561	4.85	1.1E−05	3.3E−02	0.23	9	7903755, 7903767, 7903768, **7903769**, 7903771, 7903772, 7903773, 7903774
8128459	*SIM1*	Single-minded homolog 1 (Drosophila)	NM_005068	3.87	2.3E−05	0.06	0.46	12	8128464, **8128465**
8154962	*DNAJB5*	DnaJ (Hsp40) homolog, subfamily B, member 5	NM_001135004	4.22	3.2E−05	0.07	0.64	10	8154966, **8154967**, 8154968, 8154969
8051061	*UCN*	Urocortin	NM_003353	8.46	3.6E−05	0.07	0.73	4	**8051062**
8018315	*SUMO2*	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae)	NM_006937	4.80	3.9E−05	0.07	0.79	8	8018318, **8018319**
8107356	*DCP2*	DCP2 decapping enzyme homolog (S. cerevisiae)	NM_152624	3.12	6.0E−05	0.10	1.00	16	8107358, 8107359, **8107363**
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3	NM_006187	3.00	7.2E−05	1.00	1.00	17	7958898, 7958899, 7958901, 7958903, 7958904, 7958905, 7958907, 7958908, 7958910, 7958909, 7958911, **7958912**

*Rows are sorted by increasing *P*-value for the interaction of diagnosis and exon ID.
[a]Exon probesets in bold were the most significantly differentially expressed (per gene) between diagnostic groups, and were used in SVM classification analyses.

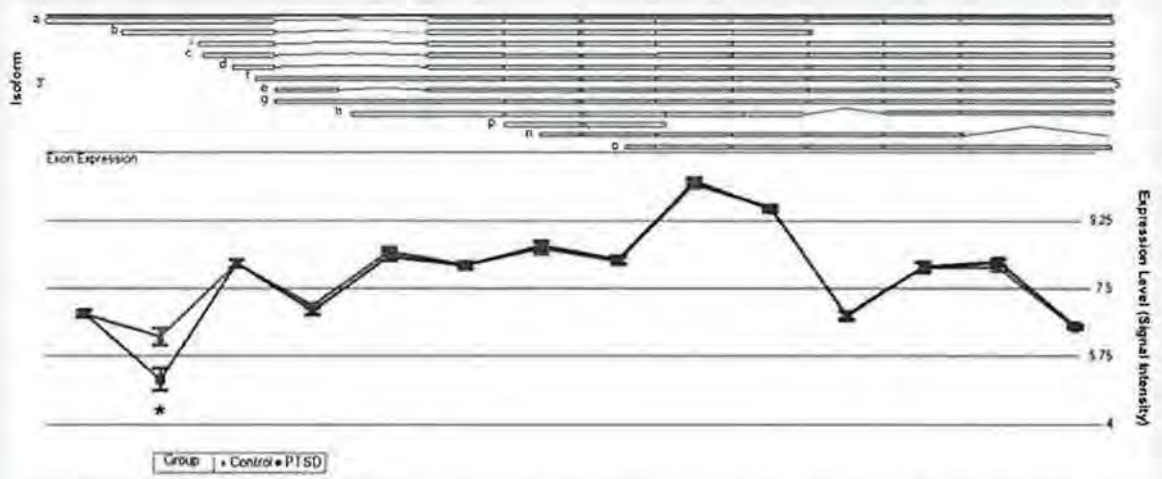

FIG. 1. Microarray-derived expression levels (ordinate) of individual exon-probes (abscissa) of suppressor of variegation 4–20 homolog 1 of *Drosophila* (*SUV420H1*) in peripheral blood mononuclear cells from eventual PTSD cases ($n = 24$; squares) and comparison subjects ($n = 24$; triangles). The interaction of diagnosis and exon ID was highly significant ($p = 1.7E-08$, Bonferroni-corrected $p = 3.4E-04$) owing to the selective down-regulation of an extended exon (probeset ID 7949933) in the 3′ end of isoform b ($*p = 0.005$) in eventual PTSD cases which occurs in the context of comparable expression levels of all other exons and isoform between groups.

as in the analyses above. For genes displaying more than one dysregulated probe between diagnostic groups, we selected the probe with the most significant between-group difference in expression level based on the *P*-values from planned comparisons. This analysis and filtering yielded 11 exons with expression differences between PTSD cases and comparison subjects (Table VI) that were then used to build and optimize SVM classifiers. The optimal SVM (identified through two-level nested 10-fold cross-validation with shrinking centroids, cost = 201, tolerance = 0.001, kernel = radial basis function, and gamma = 0.0001) comprised five of the 11 starting probes (Table VI, probes in bold font) and attained 84% accuracy in classifying those individuals in the training sample who would or would not go on to develop PTSD. We then tested the identical five-gene SVM (with the same parameters, but with no shrinkage or cross-validation) in the remaining independent test cohort (n = 10; five cases and five comparison subjects), where it yielded a diminished but reasonable 80% accuracy (higher than the accuracy observed in gene-based analyses). Among PTSD cases, three of five were correctly classified while all five comparison subjects were classified correctly. These values correspond to

TABLE VI. Exons Significantly Dysregulated in Peripheral Blood Mononuclear Cells From a Subset of Eventual PTSD Cases at Pre-Deployment and Used in Predictive SVM Classifiers[*]

Transcript cluster ID[a]	Gene symbol	Gene product	Interaction *P*	Exon ID	Fold-change	*F*	*P*-value
8040080	*RSAD2*	Radical *S*-adenosyl methionine domain containing 2	1.3E−07	8040085	2.46	10.42	2.9E−03
8133788	*PTPN12*	Protein tyrosine phosphatase, non-receptor type 12	1.8E−05	8133802	2.23	7.64	9.4E−03
8136662	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	4.8E−06	8136700	2.20	3.36	7.6E−02
8064716	*SIGLEC1*	Sialic acid binding Ig-like lectin 1, sialoadhesin	5.9E−06	8064717	1.80	15.42	4.3E−04
7958895	*OAS3*	2′,5′-oligoadenylate synthetase 3	2.7E−05	7958912	1.74	5.64	4.2E−03
7954810	*LRRK2*	Leucine-rich repeat kinase 2	8.1E−09	7954820	1.21	8.53	6.3E−03
7903765	*GSTM1*	Glutathione *S*-transferase mu 1	7.4E−05	7903769	−1.48	8.31	7.0E−03
8107356	*DCP2*	Decapping enzyme homolog (S. cerevisiae)	9.7E−05	8107363	−2.04	8.29	7.1E−03
7949931	*SUV420H1*	Suppressor of variegation 4-20 homolog 1	5.1E−06	7949933	−2.21	6.13	1.9E−02
8083282	*HPS3*	Hermansky-Pudlak syndrome 3	2.9E−06	8083291	−2.28	6.18	1.8E−02
8068740	*UMODL1*	Uromodulin-like 1	2.7E−19	8068745	−7.13	16.93	2.5E−04

[*]Rows are sorted by decreasing fold-change in eventual PTSD cases relative to non-PTSD comparison subjects.
[a]Exons of transcript cluster IDs in bold comprised the optimal 5-probe SVM classifier of eventual PTSD status identified by training and testing in independent samples.

sensitivity, specificity, positive predictive, and negative predictive values of 60%, 100%, 100%, and 71%, respectively.

DISCUSSION

A fairly consistent picture of PTSD-induced or -associated changes in peripheral blood gene expression is emerging, with immunity-related genes among the most reliably implicated biomarkers. To this picture we add new and compelling pilot data suggesting that dysregulation of immunity-related genes not only accompanies the emergence of PTSD, but precedes it. This result strongly suggests that this dysregulation is a risk factor and not simply a consequence of PTSD. Yet, immune-gene dysregulation may be only one piece of the biological puzzle of PTSD susceptibility, as many genes comprising the best-performing PTSD-predictive classifiers were not immune-system genes, and these other genes had highly disparate functions.

Collectively, profiles of dysregulated genes in immune and other pathways may serve as potent risk indicators upon which early intervention and prevention efforts may ultimately be based. *To wit*, we were able to construct and validate two panels of blood-based PTSD risk-predictive biomarkers that ranged in accuracy from 70% to 80% in independent (albeit small) replication samples. Despite our relatively small sample size and the additional levels of correction for multiple-testing required for exon analyses, a number of differentially expressed exons surpassed stringent criteria for declaring statistical significance. Additionally, the exon-based predictive classifier appeared to perform better than the gene-based predictive classifier. Taken together, these findings suggest that exon expression may be more reliable and biologically informative than gene expression (which reflects the average expression of all transcript isoforms of a particular cluster).

It is important to note that these classifiers employed decision-rules based solely on mRNA expression levels. Possibly, more accurate classification models can be constructed in the future by taking into account additional known predictors of PTSD, such as family history, personality traits, pre-existing mental disorders [Koenen et al., 2003a,b], and other factors not necessarily related to gene expression. Alternatively, risk factors such as childhood exposure to trauma [van Zuiden et al., 2012] might actually be associated with or interact with alterations in pre-deployment mRNA-expression profiles. The present study was unable to account for childhood exposure to trauma or other such factors, but future efforts to construct predictive models should seek to incorporate such data. Further precision in measuring the amounts and types of mRNA isoforms present in peripheral blood (e.g., by further analyses of exon-level expression, or by quantitation of distinct alternatively spliced isoforms through RNAseq or exon–exon junction-probing microarrays) will undoubtedly also facilitate the construction of more accurate classifiers. Nevertheless, a single predictive classifier of PTSD (no matter how precisely constructed) may never perform with 100% accuracy, which is why it will be essential to pursue (in larger samples) those characteristics of either the subjects or the data that would determine for whom such a classifier works. Of equal interest is the possibility that, despite similar phenotypic manifestations of PTSD, there are two or more unique biomarker profiles that predict the same phenotypic outcome. In fact, etiologic heterogeneity may be a hallmark of complex disorders including PTSD, so it may not be possible to identify a single "one-size-fits-all" biomarker profile of the susceptibility toward the disorder. Thus, in the future, distinct predictive biomarker classifiers may be required to account for disorder stratification and correctly classify biologically or phenotypically separate sets of subjects at highest risk of developing PTSD. Another distinct possibility is that for some eventual cases of PTSD there is no blood-based pre-trauma biomarker signature of increased susceptibility to be found. We are currently investigating each of these possibilities further.

Because of our relatively small sample size and the severe corrections for multiple-testing required when examining the entire transcriptome, we did not detect individual gene-expression differences in eventual PTSD cases that surpassed stringent criteria for declaring statistical significance. As such, the focus of our efforts and interpretations has been on groups of genes, either in regard to their biological annotations or their collective ability to identify PTSD cases. Nevertheless, one gene identified here as predictive of PTSD emergence (*RPL24*) is notable in that it was also identified as a diagnostic biomarker of PTSD in a prior blood-based gene expression study by Mehta and Binder [2012]. Interestingly, we found that this gene was significantly down-regulated at pre-deployment among Marines who would later go on to develop PTSD, whereas Mehta et al., found this gene to be up-regulated in current PTSD sufferers. If this observation can be confirmed by additional work, it suggests that the down-regulation of *RPL24* at baseline may signal heightened susceptibility for the disorder which is then accompanied by a concomitant increase and over-expression of this gene after exposure to the precipitating trauma and subsequent development of PTSD symptoms. The majority of genes that we found to be dysregulated at baseline in eventual PTSD cases do not appear in other post-trauma studies to be either significantly up- or down-regulated in established PTSD cases, suggesting that the expression levels of these genes simply signify a risk state but do not necessarily bear on the presentation of the disorder once trauma has been experienced. Our results must be validated using another more sensitive mRNA-quantification technique such as qRT-PCR, but beyond this, replication in other well-powered longitudinal studies of subjects at high risk for trauma will prove crucial for more definitively implicating particular genes as risk indicators.

The present pilot study broadens the search for pre-deployment biomarkers for PTSD vulnerability beyond that of previous work [e.g., van Zuiden et al., 2012]. To our knowledge, this was the first study to search transcriptome-wide for patterns of gene- and exon-expression that distinguished future PTSD cases from non-PTSD comparison subjects. The present study is also unique because it employed a data-driven machine-learning approach for identifying the transcripts that, collectively, were most predictive of future PTSD status, many of which had not previously been associated with PTSD. Taken together, these two strategies are useful for identifying exons, genes, and pathways that potentially serve as biomarkers and play a role in the etiology of PTSD, but that may have been overlooked by other approaches focusing on well-established candidate genes.

This work must be considered in the context of its limitations. Foremost among these may be the observation of an increased pre-

deployment CAPS score among future PTSD cases. A closer examination of this finding revealed that this difference was driven by the "D" subscale of the CAPS measure, reflecting an increased reporting of symptoms of hyper-arousal among future cases. Because of this limitation, it cannot be determined unequivocally whether the present study has detected true biological vulnerability, pre-clinical changes associated with PTSD, or (more likely) some combination of these factors. Conclusions about the origins of the blood-based biomarker signals (vulnerability vs. preclinical state) could be strengthened in future studies by controlling for the severity of prior trauma exposure, or better yet, by examining pre-deployment gene expression in trauma-naïve subjects. Nevertheless, we maintain that the design of our study lends itself to the potential development of a predictive biomarker with some clinical utility; one that potentially can be used to determine who is at increased risk for emergent PTSD among a group of real-world service members who will undoubtedly have mixed and incomplete records of trauma exposure and may even manifest signs of pre-clinical disorder.

Regardless of the preliminary state of our conclusions regarding individual genes, our work makes clear that genes involved in cellular immunity are reliably and disproportionately represented among those that are dysregulated (mostly up-regulated) in our sample of eventual PTSD cases. This finding is consistent with evidence for dysfunctional cellular immune processes in individuals with PTSD, which we recently reviewed in depth [Baker et al., 2012b]. Our review of the collective evidence suggests that systemic inflammation and deleterious health consequences in PTSD are strongly linked. Given this evidence, treatment strategies to reduce inflammation that target biobehavioral factors may be of value to pursue.

In conclusion, as the development of PTSD following initial trauma exposure is quite variable and unpredictable, we sought to identify readily assessable biomarkers of risk and resilience based on evaluations of blood-based gene expression among soon-to-be-deployed Marines participating in the MRS. Our analyses converged on the immune-system as the most reliably dysregulated biological process characterizing high-risk individuals; however, numerous other genes not strictly related to cellular immunity also appear to be differentially expressed at baseline in individuals who develop PTSD, and these genes contribute much to our blood-based prediction models of the disorder's emergence. If biomarkers related to PTSD risk and resilience (such as the panels of genes and exons identified here) can be validated in additional cohorts and prospective studies, they may help to confidently identify which individuals are at the highest risk in real-world scenarios. These efforts may lead to more effective primary prevention protocols, which would be particularly important in groups such as these Marines for whom it is known in advance that exposure to serious trauma is highly likely. This may also prove highly relevant for first-responders, such as police, fire, and emergency medical teams, for whom a regular part of their job is also exposure to potentially traumatic situations. Further work correlating pre- and post-deployment differences in gene expression among PTSD cases and unaffected comparison subjects would also constitute a major advance in the effort to identify the biological mechanisms of this disorder and potentially develop diagnostic biomarkers that can serve as useful adjuncts to the prevailing gold-standard behavioral diagnostic systems [Brewin et al., 2000; Ozer et al., 2003].

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Original Investigation

Association Between Traumatic Brain Injury and Risk of Posttraumatic Stress Disorder in Active-Duty Marines

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IMPORTANCE Whether traumatic brain injury (TBI) is a risk factor for posttraumatic stress disorder (PTSD) has been difficult to determine because of the prevalence of comorbid conditions, overlapping symptoms, and cross-sectional samples.

OBJECTIVE To examine the extent to which self-reported predeployment and deployment-related TBI confers increased risk of PTSD when accounting for combat intensity and predeployment mental health symptoms.

DESIGN, SETTING, AND PARTICIPANTS As part of the prospective, longitudinal Marine Resiliency Study (June 2008 to May 2012), structured clinical interviews and self-report assessments were administered approximately 1 month before a 7-month deployment to Iraq or Afghanistan and again 3 to 6 months after deployment. The study was conducted at training areas on a Marine Corps base in southern California or at Veterans Affairs San Diego Medical Center. Participants for the final analytic sample were 1648 active-duty Marine and Navy servicemen who completed predeployment and postdeployment assessments. Reasons for exclusions were nondeployment (n = 34), missing data (n = 181), and rank of noncommissioned and commissioned officers (n = 66).

MAIN OUTCOMES AND MEASURES The primary outcome was the total score on the Clinician-Administered PTSD Scale (CAPS) 3 months after deployment.

RESULTS At the predeployment assessment, 56.8% of the participants reported prior TBI; at postdeployment assessment, 19.8% reported sustaining TBI between predeployment and postdeployment assessments (ie, deployment-related TBI). Approximately 87.2% of deployment-related TBIs were mild; 250 of 287 participants (87.1%) who reported posttraumatic amnesia reported less than 24 hours of posttraumatic amnesia (37 reported ≥24 hours), and 111 of 117 of those who lost consciousness (94.9%) reported less than 30 minutes of unconsciousness. Predeployment CAPS score and combat intensity score raised predicted 3-month postdeployment CAPS scores by factors of 1.02 ($P < .001$; 95% CI, 1.02-1.02) and 1.02 ($P < .001$; 95% CI, 1.01-1.02) per unit increase, respectively. Deployment-related mild TBI raised predicted CAPS scores by a factor of 1.23 ($P < .001$; 95% CI, 1.11-1.36), and moderate/severe TBI raised predicted scores by a factor of 1.71 ($P < .001$; 95% CI, 1.37-2.12). Probability of PTSD was highest for participants with severe predeployment symptoms, high combat intensity, and deployment-related TBI. Traumatic brain injury doubled or nearly doubled the PTSD rates for participants with less severe predeployment PTSD symptoms.

CONCLUSIONS AND RELEVANCE Even when accounting for predeployment symptoms, prior TBI, and combat intensity, TBI during the most recent deployment is the strongest predictor of postdeployment PTSD symptoms.

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Traumatic brain injury (TBI) is common. According to a 2010 Centers for Disease Control and Prevention report,[1] at least 1.7 million Americans annually sustain TBI. A significant number of injury survivors join more than 5 million (approximately 2%) Americans already living with TBI-related disabilities, which comprise a wide range of medical, cognitive, emotional, and behavioral impairments.[2,3] The estimated economic burden of TBI in the United States in 2000, prior to initiation of the Iraq and Afghanistan conflicts, was approximately $60 billion annually.[4]

Pervasive use of improvised explosive devices (IEDs), rocket-propelled grenades, and land mines in the Iraq and Afghanistan theaters has brought TBI and its effect on health outcomes into public awareness.[5-7] Blast injuries have been deemed signature wounds of these conflicts, with an estimated 52% of deployment-related TBI cases caused by IEDs.[5,8] Of Operations Enduring Freedom, Iraqi Freedom, and New Dawn service members, approximately 10% to 20% reported mild TBI or concussion, and nearly 60% of those reported exposure to more than 1 blast.[9-11]

War-related TBI is not new, having become prevalent during World War I and remaining medically relevant in World War II and beyond.[12,13] Medicine's past attempts to disentangle the pathophysiology of war-related TBI parallels current lines of inquiry and highlights limitations in methods and attribution of the cause of symptoms, be it organic, psychological, or behavioral.[14] Thus far, cross-sectional data from the Operations Enduring Freedom, Iraqi Freedom, and New Dawn conflicts reveal significantly higher rates of psychiatric symptoms, including posttraumatic stress disorder (PTSD), in deployed than in nondeployed service members.[10,15,16] Moreover, self-reported TBI and PTSD symptoms show considerable overlap.[17] Symptoms of PTSD are reported at approximately double the rate by service members who show positive results on screening for mild TBI in comparison with those who report no TBI.[9,18] These cross-sectional studies limit causal inference and stress the need for longitudinal data to define further the contribution of war-related TBI to PTSD. Using data from the Marine Resiliency Study, a prospective, longitudinal study of infantry Marines,[19] we examined whether deployment-related TBI predicts PTSD symptom severity when accounting for combat intensity and predeployment characteristics.

Methods

Study Design and Participants

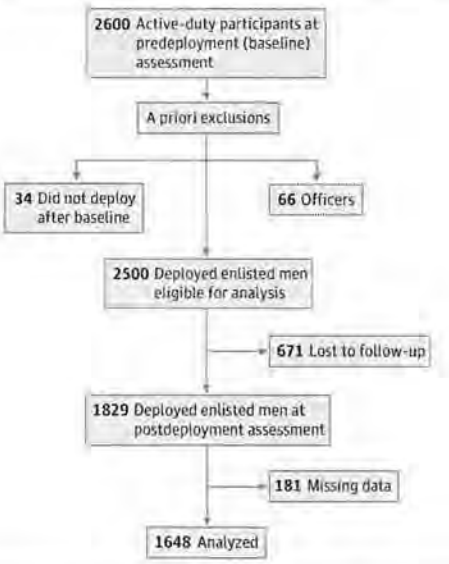
We extracted data from a longitudinal study of 2600 active-duty Marine and Navy servicemen from 4 infantry battalions of the First Marine Division stationed in southern California. Assessments were conducted between July 14, 2008, and May 24, 2012, and were centered on the deployments of each battalion. Servicemen were evaluated approximately 1 month before a 7-month deployment to Iraq or Afghanistan, 1 week after deployment, and 3 and 6 months after deployment. For this study, we used data collected at predeployment, as well as 1 week and 3 months after deployment. Data from the 6-month postdeployment evaluation were not analyzed because of reduced follow-up rates. This study was approved by the institutional review boards of the University of California, San Diego; the Veterans Affairs San Diego Research Service; and the Naval Health Research Center (University of California, San Diego, and Veterans Affairs San Diego Research Service approval 070533), and written informed consent was obtained from all participants.[19] Participants received financial compensation for each study visit in which a blood draw occurred (ie, predeployment, 3-months, and 6-months postdeployment).

The **Figure** shows the sampling composition and exclusions. Of the 2600 servicemen assessed at predeployment, 34 did not deploy and were excluded a priori as well as 66 officers who were significantly older ($P < .001$) and had lower Combat Experience Scale (CES) scores ($P < .001$) than enlisted participants. Forty-five of the 66 officers (68%) were missing cognitive ability scores on a military enlistment test (Armed Forces Qualification Test [AFQT]), an important variable associated with resilience.[20] The 32% of officers with available AFQT scores scored significantly higher than current enlisted participants ($P < .001$). Of the remaining 2500 individuals, 1829 completed the 3-month postdeployment assessment. Of these, 181 were excluded for missing data on measures used in the present analysis. The final analytic sample included 1648 participants.

Measures

Complete Marine Resiliency Study methods are described elsewhere.[19] Measures relevant to the present study are described here. Posttraumatic stress symptoms were assessed using the Clinician-Administered PTSD Scale (CAPS),[21] a 17-item criterion standard, structured diagnostic interview developed by the National Center for PTSD,[22-24] administered before deployment and 3 months after deployment. We captured

Figure. Flowchart



Longitudinal sample composition and reasons for exclusion.

the worst lifetime event in 2351 of the 2600 servicemen (90.4%) assessed at predeployment. Interrater reliability for the CAPS total score was high (intraclass correlation coefficient, 0.99).[25] Our outcome variable was 3-month postdeployment CAPS total score (possible range, 0-136), with higher scores indicating greater symptom severity. Posttraumatic stress disorder was defined as a score of 65 or greater,[24] partial PTSD as scores of 40 to 64, healthy/minimally symptomatic as scores of 1 to 39, and no symptoms as a score of 0.[23]

We inquired via face-to-face interview about any lifetime head injuries sustained before the index deployment and injuries sustained between the predeployment and 3-month postdeployment assessments. Participants were asked whether they sustained a head injury from a blast or explosion, vehicular accident, fragment or bullet wound above the shoulder, fall, blunt object, being rendered unconscious by another person, or by any other means. Probable TBI was any head injury resulting in self-reported loss of consciousness (LOC) or altered mental status (ie, dazed, confused, "seeing stars," and/or posttraumatic amnesia [PTA]) immediately afterward or upon regaining consciousness.[26-28] The time between predeployment and postdeployment assessments was broader than the deployment; thus, nondeployment TBIs sustained between assessment dates were included in analyses to account for potential effects on PTSD symptoms.[29] For parsimony, we labeled all TBIs experienced between predeployment and postdeployment assessments as *deployment-related* TBI, realizing that few were experienced outside of deployment and that some TBIs experienced before the study's predeployment assessment were acquired during a prior deployment.

Combat intensity was measured using a modified 16-item, 5-point Likert version of the Deployment Risk and Resilience Inventory[30,31] CES. The CES was administered during a brief session conducted 1 week after deployment. Response items ranged from 0 (never) to 4 (daily or almost daily) and were summed to yield a total score. Possible total CES scores range from 0 to 64, with higher total scores indicating more intense combat.

The AFQT,[20] a military enlistment aptitude test of general cognitive ability, has been negatively associated with PTSD outcomes.[32] The AFQT scores were obtained from the Career History Archival Medical and Personnel System database maintained by the Naval Health Research Center and were included as a covariate along with battalion, age, and rank. Self-reported race and ethnicity have been shown to vary with PTSD and were also entered as covariates.[33,34]

Statistical Analysis

All continuous predictors, except predeployment CAPS scores, were centered before analysis. A priori χ^2 tests showed battalion differences in deployment and TBI characteristics (Supplement [eTable 1]). We corrected for these and other unknown battalion differences, such as training schedules, timing of assessments, group leadership, and cohesion, by including battalion as a covariate. Battalion, TBI, race, and ethnicity were dummy-coded with the following reference groups: battalion 1, no TBI, white, and non-Hispanic. Analyses were conducted using statistical software package R, version 2.15.3.[35]

Predeployment differences between participants in the final sample and nonparticipants (ie, servicemen assessed at predeployment only or excluded otherwise) were tested using a paired, 2-tailed *t* test, exact conditional test of proportions, or χ^2, as appropriate. Differences in predeployment CAPS scores were analyzed using zero-inflated negative binomial regression (ZINBR) because of overdispersion.

The CAPS outcome scores were positively skewed, overdispersed, and had an excess of zero scores (Supplement [eFigure]). Zero-inflated negative binomial regression was the best-fitting model[36] for our data (Supplement [eAppendix and eTable 2]) and was used to test effects of predeployment PTSD symptoms, combat intensity, and prior and deployment-related TBI on 3-month postdeployment PTSD symptoms. The ZINBR model accounts for a positively skewed integer-valued distribution with a high proportion of zero scores.[37] This model assumes that our sample contains a mixture of participants whose CAPS outcome scores are generated by the standard negative binomial distribution and those who have zero probability of a CAPS outcome score greater than zero (eg, resulting from nontraumatic CAPS event and possible genetic or biological resilience). An observed CAPS score of zero could come from either group. Zero-inflated negative binomial regression uses maximum likelihood to model outcomes via 2 component models: logistic regression (the zero model) predicts the probability of a CAPS outcome score of zero, and negative binomial regression (the count model) predicts change in CAPS score. Throughout this article we refer to predicting the odds of a zero vs nonzero outcome as the zero model and predicting nonzero outcomes as the count model.

Model estimates and predeployment symptom severity, combat intensity, and TBI were used to predict postdeployment symptom severity. Additional ZINBR models assessed the effects of TBI-related attributes, including injury severity (mild vs moderate/severe), time since most recent TBI, single vs multiple deployment-related TBIs, and group comparisons among deployment-related TBIs with LOC, TBI without LOC, and no deployment-related TBI.

Results

Sample Characteristics

Predeployment sample characteristics were similar to demographics of other deployed service members (**Table 1**).[38] Participants were younger (mean [SD] age, 22.4 [3.3] vs 23.0 [3.4] years), more likely to be junior enlisted (74.1% vs 62.2%), and were less likely to have had prior deployments (45.3% vs 62.0%) compared with nonparticipants. Approximately 31.8% of participants were married. Participants had lower childhood trauma scores (39.8 [13.2] vs 41.6 [14.8]), and better predeployment 12-item Short-Form Health Survey physical health component scores (53.9 [6.3] vs 52.6 [6.8]) than nonparticipants. Participants and nonparticipants did not differ significantly in other demographic and predeployment factors, including AFQT scores, depression, anxiety, CAPS scores, 12-item Short-Form Health Survey mental health scores, and predeployment TBI rates.

Table 1. Comparisons of Predeployment Characteristics Between Participants and Nonparticipants

Predeployment Characteristic	Nonparticipants (n = 852)[a]	Participants (n = 1648)	P Value
Age, mean (SD), y	23.0 (3.4)	22.4 (3.3)	<.001
Race/ethnicity, %[b]			
Hispanic	25.7	22.6	.10
White	82.3	84.9	.13
Educational level ≤high school, %	67.4	69.5	.27
Married, %	40.7	31.8	<.001
Years in military service, mean (SD)	3.3 (2.6)	2.7 (2.7)	<.001
Rank E1-E3 vs E4-E9, %	62.2	74.1	<.001
Prior deployment, %	62.0	45.3	<.001
Prior TBI, %	52.8	56.8	.06
Assessment scale scores, mean (SD)			
AFQT	58.1 (19.1)	59.8 (19.2)	.07
Childhood trauma	41.6 (14.8)	39.8 (13.2)	.003
SF-12 mental health	49.0 (9.4)	49.64 (8.7)	.14
SF-12 physical health	52.6 (6.8)	53.9 (6.3)	<.001
CAPS[c]	15.6 (16.6)	14.9 (14.8)	.38
Beck Anxiety Index Scale[d]	7.2 (8.6)	6.8 (7.6)	.35
Beck Depression Inventory Scale[e]	6.8 (8.2)	6.6 (7.5)	.69

Abbreviations: AFQT, Armed Forces Qualification Test; CAPS, Clinician-Administered PTSD [posttraumatic stress disorder] Scale; E1-E3, junior enlisted; E4-E9, senior enlisted; SF-12, 12-item Short-Form Health Survey; TBI, traumatic brain injury.

[a] Nonparticipants were defined as enlisted servicemen who deployed but completed only the predeployment assessment or were missing data required for the final model.

[b] African Americans constituted approximately 4.3% of participants and 5.7% of nonparticipants.

[c] Predeployment CAPS median score (interquartile range [IQR]) was 11 (21 − 3 = 18) for nonparticipants and 11 (21 − 4 = 17) for participants.

[d] Beck Anxiety Index median score (IQR) was 4 (11 − 1 = 10) for nonparticipants and 4 (10 − 1 = 9) for participants.

[e] Beck Depression Inventory median score (IQR) was 4 (10 − 1 = 9) for both nonparticipants and participants.

Table 2 reports the final sample characteristics. Of the total number of respondents, 56.8% reported probable TBI before the index (ie, most recent) deployment. At the 3-month postdeployment assessment, 40 of the participants (2.4%) had CAPS scores of 65 or more, and 327 individuals (19.8%) reported sustaining TBI after predeployment, with 295 (17.9%) reporting TBI during the index deployment. Of the 32 participants reporting nondeployment TBI between predeployment and 3-month postdeployment assessments, 2 sustained TBI after predeployment but before the index deployment, and 24 sustained TBI after their index deployment but before their follow-up assessment; the event timing of 6 TBIs could not be verified. There were no significant differences between deployment TBI and nondeployment TBI sustained between predeployment and postdeployment on model outcomes; thus, nondeployment TBIs were included in the main analysis. Mean time since most recent TBI was 200 (126) days. Of the 327 individuals who sustained TBI after the predeployment assessment, 112 participants (34.3%) reported more than 1 TBI, and 285 TBIs (87.2%) were categorized as probably mild[39]; 208 of 327 individuals (63.6%) reported alteration of consciousness without LOC, 250 of 287 (87.1%) who reported PTA indicated less than 24 hours of PTA (37 reported >24 hours), and 111 of 117 participants (94.9%) who lost consciousness reported less than 30 minutes of LOC. Severity of 4 TBIs (1.2%) was unknown. Participants who sustained TBI after the predeployment assessment were more likely than others to have had prior TBI and reported more severe predeployment PTSD symptoms and greater combat intensity during their index deployment.

Zero-Inflated Negative Binomial Regression

Results of ZINBR are reported in Table 3. A significant main effect reflected a predictor's association with postdeployment CAPS scores given a predeployment CAPS score of zero, mean scores on all other continuous predictors, and reference group membership for categorical predictors. Significant interactions out of all possible tested are reported.

Zero Model: Predicting Absence of PTSD Symptoms

Logistic regression was used to predict probability of a 3-month postdeployment CAPS score of zero. Coefficients were exponentiated and interpreted as odds of a zero CAPS score. The zero model intercept reflects a 27.1% base probability of having a postdeployment CAPS score of zero given the participant was white, non-Hispanic, from battalion 1, had no predeployment or deployment TBI, had a predeployment CAPS score of zero, and had average scores on all other continuous predictors.

For the zero model, deployment-related TBIs were collapsed across severity because the small number of moderate/severe TBIs caused problems with model convergence. Unit increases in predeployment CAPS scores decreased the odds

Table 2. Descriptive Statistics for Participants Reporting TBI vs No TBI Sustained After Predeployment Assessment

Characteristic	Mean (SD)		
	No TBI (n = 1321)	TBI (n = 327)[a]	All Participants (N = 1648)
Predeployment variable, %			
Hispanic	22.0	25.1	22.6
White	84.9	85.0	84.9
Rank E1-E3	74.5	72.2	74.1
Prior TBI	54.5	66.1	56.8
In Battalion 1	15.5	11.9	14.8
Age, y	22.5 (3.4)	22.2 (2.8)	22.4 (3.3)
AFQT	60.4 (19.2)	57.4 (19.3)	59.8 (19.2)
CAPS score[b]	14.3 (14.6)	17.4 (15.2)	14.9 (14.8)
Deployment variable			
Combat experience score[c]	10.5 (8.7)	22.4 (13.4)	12.9 (10.9)
3-mo Postdeployment variable			
CAPS score %[d]			
Asymptomatic, score 0	22.3	4.9	18.8
Minimally symptomatic, scores 1-39	70.2	70.0	70.2
Partial PTSD, scores 40-64	6.1	18.7	8.6
PTSD, scores ≥65	1.4	6.4	2.4

Abbreviations: AFQT, Armed Forces Qualification Test; CAPS, Clinician-Administered PTSD Scale; E1-E3, junior enlisted; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a] Of the 327 individuals who reported deployment-related TBI, 285 (87.2%) reported mild symptoms; 208 (63.6%) reported alteration without loss of consciousness, 250 of 287 (87.1%) with posttraumatic amnesia reported less than 24 hours of posttraumatic amnesia, and 111 of 117 (94.9%) participants who lost consciousness reported less than 30 minutes' loss of consciousness. Approximately 34.3% reported more than 1 TBI, and mean (SD) time since most recent TBI was 200 (126) days.

[b] Predeployment CAPS median score (interquartile range [IQR]) was 10 (20 − 4 = 16) for no TBI, 14 (25 − 5 = 20) for TBI, and 11 (21 − 4 = 17) for all participants.

[c] Combat experience median score (IQR) was 8 (14 − 4 = 10) for no TBI, 20 (32 − 11 = 21) for TBI, and 10 (17 − 5 = 12) for all participants.

[d] Postdeployment CAPS median score (IQR) was 10 (21 − 2 = 19) for no TBI, 24 (40 − 12 = 28) for TBI, and 12 (26 − 4 = 22) for all participants.

of an outcome (ie, postdeployment) CAPS score of zero by a factor of 0.92 (7.7%; $P < .001$). Unit increases in combat intensity reduced the odds by a factor of 0.96 (3.6%; $P < .001$). Prior TBI reduced the odds of having an outcome CAPS score of zero by a factor of 0.65 (35.5%; $P < .01$), and deployment-related TBI reduced the odds by a factor of 0.34 (66.1%; $P < .01$). There were no effects of TBI with vs without LOC, time since most recent TBI, or single vs multiple deployment-related TBI on the absence of postdeployment symptoms.

Count Model: Predicting PTSD Symptom Severity

The count model predicted the postdeployment CAPS scores being generated from a negative binomial distribution. Exponentiated coefficients of the counts model represent multiplicative change in predicted CAPS score per unit change in a given predictor. The intercept reflects a predicted postdeployment CAPS score of 12.54 given the participant was white, non-Hispanic, from battalion 1, had no TBI, had a predeployment CAPS score of zero, and had average scores on all other continuous predictors.

Predeployment CAPS score and combat intensity score raised the predicted 3-month postdeployment CAPS score by factors of 1.02 (1.9%; $P < .001$) and 1.02 (1.5%; $P < .001$) per unit increase, respectively. Prior (ie, pre-index deployment) TBI raised the predicted CAPS outcome score by a factor of 1.08 (7.5%), but the effect was not significant ($P < .08$). Deployment-related mild TBI raised the predicted CAPS score by a factor of 1.23 (22.6%; $P < .001$), and deployment-related moderate/severe TBI raised the predicted CAPS score by a factor of 1.71 (70.5%; $P < .001$). Dividing the estimated coefficients for deployment-related TBI by combat intensity yielded the equivalent of a 14.0-point increase in combat intensity for participants reporting mild TBI, and a 36.6-point increase for those reporting moderate/severe TBI. There were no effects of deployment-related TBI with vs without LOC, time since recent TBI, or single vs multiple TBI on postdeployment symptom severity.

There was a relatively small interaction effect that accounted for less than 1% change in 3-month postdeployment CAPS score. Unit increases in AFQT increased the predicted CAPS score by 0.8% ($P < .001$), but this effect was reduced by roughly two-thirds in participants with predeployment TBI ($P < .02$).

The overall effects of predeployment symptoms, combat intensity, and TBI on postdeployment PTSD symptoms were confirmed using logistic regression to determine the effects of the same predictors as in the final ZINBR model on the categorical outcome of PTSD vs no PTSD at 3-month postdeployment assessment (Supplement [eMethods, eResults, and eTable 3]).

Predictions

Predeployment CAPS scores, combat intensity, and deployment-related mild TBI were used to predict the probability that

Table 3. Zero-Inflated Negative Binomial Regression Predicting Postdeployment PTSD Symptoms

Model	Variable	Estimate (SE)	*P* Value	Predicted CAPS Total[a]	Ratio (95% CI)
Count	(Intercept)	2.53 (0.06)	<.001	12.54	(11.10-14.17)
	Battalion 2	−0.03 (0.06)	.65		0.97 (0.86-1.00)
	Battalion 3	−0.05 (0.06)	.46		0.96 (0.85-1.08)
	Battalion 4	0.13 (0.07)	.06		1.14 (0.10-1.31)
	CAPS score, predeployment	0.02 (0.00)	<.001		1.02 (1.02 -1.02)
	AFQT	0.01 (0.00)	<.001		1.01 (1.01-1.01)
	TBI, predeployment	0.07 (0.04)	.07		1.08 (0.99-1.16)
	AFQT × TBI, predeployment	−0.0 (0.00)	.02		1.00 (0.99-1.00)
	Combat Experience Score	0.01 (0.00)	<.001		1.02 (1.01-1.02)
	Mild TBI, deployment[b]	0.20 (0.05)	<.001		1.23 (1.11-1.36)
	Moderate/severe TBI, deployment	0.53 (0.11)	<.001		1.71 (1.37-2.12)
Zero	(Intercept)	−0.10 (0.25)	<.001	27.10%	(18.60%-37.69%)
	Battalion 2	0.93 (0.24)	<.001		2.52 (1.60-4.06)
	Battalion 3	0.63 (0.25)	.01		1.87 (1.14-3.07)
	Battalion 4	0.33 (0.29)	.26		1.39 (0.79-2.45)
	CAPS score, predeployment	−0.08 (0.01)	<.001		0.92 (0.90-0.94)
	TBI, predeployment	−0.44 (0.15)	.003		0.64 (0.48-0.86)
	Combat Experience Score	−0.04 (0.01)	<.001		0.96 (0.94-0.98)
	TBI, deployment[b,c]	−1.08 (0.30)	<.001		0.34 (0.19-0.62)

Abbreviations: AFQT, Armed Forces Qualification Test; CAPS, Clinician-Administered PTSD Scale; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a] For the zero model, base probability (%) of a predicted CAPS total score, 0.

[b] There were no significant differences between deployment and nondeployment TBI sustained between predeployment and postdeployment assessments (n = 32). Thus, nondeployment TBI was included in the analysis to account for any potential effects on PTSD outcomes.[27]

[c] For the zero model, deployment-related TBIs were collapsed across severity because of the small number of moderate/severe TBIs causing problems with model convergence.

3-month postdeployment CAPS scores would fall within defined symptom ranges for partial PTSD and PTSD while holding all other variables constant (**Table 4**). Predeployment CAPS scores used for prediction were 0 (no symptoms), 19 (healthy/minimally symptomatic; range, 1-39), 52 (partial PTSD; range, 40-64), and 65 (PTSD; scores ≥65).[23] Low and high combat intensity were defined as CES scores of 5 (25th percentile) and 19 (75th percentile), respectively.

Based on study outcomes, participants with no predeployment symptoms, low combat intensity, and no deployment-related TBI were ascertained to have a predicted 3-month postdeployment CAPS score of 7.23, with less than 1% probability of partial PTSD or PTSD. Deployment-related mild TBI raised the predicted CAPS score slightly to 11.45, with 1.5% probability of partial PTSD.

Participants who were minimally symptomatic before deployment had low combat intensity, and those with no TBI had less than 4% predicted probability of postdeployment partial PTSD (3.2%) and PTSD (0.2%). High combat intensity increased predicted rates to 6.9% for partial PTSD and 0.8% for PTSD. In addition, deployment-related mild TBI nearly doubled outcome rates to 12.4% for partial PTSD and 2.4% for PTSD.

Compared with the minimally symptomatic group, participants whose predeployment CAPS scores met the criteria for partial PTSD or PTSD had higher predicted probabilities of postdeployment PTSD at 3 months, even with low combat intensity (>6%). Higher combat intensity increased predicted PTSD rates for those who reported partial symptoms before deployment (12.3%), and deployment-related mild TBI further increased predicted PTSD rates for this group (21.1%).

Discussion

As expected, both predeployment psychiatric symptoms and combat intensity significantly predicted postdeployment PTSD symptoms. Predeployment psychiatric conditions have been deemed a risk factor for PTSD and other mental health problems during deployment.[40] Likewise, prior psychological trauma[16,41] and extensive combat exposure[15,16,42,43] may increase PTSD risk after combat deployment.

Independent of the above effects, TBI sustained before the index deployment was associated with more severe postdeployment PTSD symptoms. According to our model, deployment-related TBIs nearly double the likelihood of postdeployment PTSD for participants who reported minimal to no symptoms before deployment. Probability of postdeployment PTSD was greatest for participants reporting prior psychiatric symptoms and deployment-related TBI. However, of the 16 participants with predeployment PTSD, 8 considerably improved (postdeployment CAPS range, 0-35) and 3 slightly improved (range, 50-78), whereas 3 worsened (range, 78-94). In contrast to those with improved symptoms, participants with persistent symptoms reported higher combat intensity (mean score, 22.7 vs 8.4) and 2 of the 3 reported deployment-related TBI. These findings parallel reported symptom trajectories for deployed service members in which 8% showed improvement in PTSD symptoms and 2.2% showed continuation of severe symptoms.[44]

Prior cross-sectional studies have also reported associations between TBI and PTSD,[45,46] although injury severity may govern the association.[47,48] Higher morbidity and use of medical services are associated with severe TBI, whereas mental

Table 4. Predictions of Postdeployment CAPS Scores and Outcome Probabilities

Predeployment Symptom Severity (N = 1648)[a]	Combat Intensity[b]	Mild Deployment TBI	Predicted Mean Postdeployment CAPS Score (95% CI)	% Predicted Probability of Partial PTSD (95% CI)[c]	% Predicted Probability of PTSD (95% CI)[c]
No symptoms (n = 243)	Low	No	7.23 (6.10-8.36)	0.38 (0.27-0.51)	0.01 (0.00-0.02)
		Yes	11.45 (10.18-12.72)	1.50 (1.28-1.75)	0.05 (0.01-0.10)
	High	No	10.29 (9.00-11.58)	1.35 (1.13-1.58)	0.04 (0.01-0.09)
		Yes	14.95 (13.90-16.00)	3.88 (3.51-4.27)	0.26 (0.16-0.36)
Minimally symptomatic (n = 1283)	Low	No	14.17 (13.43-14.91)	3.22 (2.87-3.57)	0.18 (0.10-0.27)
		Yes	18.63 (18.09-19.18)	7.12 (6.63-7.63)	0.77 (0.61-0.95)
	High	No	18.13 (17.47-18.80)	6.93 (6.43-7.43)	0.75 (0.59-0.93)
		Yes	23.21 (22.79-23.63)	12.44 (11.80-13.11)	2.37 (2.08-2.68)
Partial PTSD (n = 106)	Low	No	29.40 (29.13-29.67)	19.01 (18.27-19.79)	6.21 (5.74-6.69)
		Yes	36.25 (36.05-36.45)	24.13 (23.30-24.96)	12.35 (11.72-13.01)
	High	No	36.19 (35.96-36.42)	24.09 (23.25-24.92)	12.33 (11.70-12.98)
		Yes	44.52 (44.34-44.71)	27.12 (26.25-27.99)	21.08 (20.30-21.88)
PTSD (n = 16)[d]	Low	No	37.89 (37.68-38.09)	24.97 (24.10-25.83)	14.02 (13.35-14.96)
		Yes	46.55 (46.36-46.75)	27.44 (26.57-28.32)	23.27 (22.47-24.11)
	High	No	46.54 (46.34-46.73)	27.42 (26.54-28.29)	23.27 (22.44-24.09)
		Yes	57.14 (56.95-57.33)	27.32 (26.45-28.19)	34.36 (33.44-35.29)

Abbreviations: CAPS, Clinician-Administered PTSD Scale; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a] CAPS scores used for prediction were no symptoms (score, 0), healthy/minimally symptomatic (median score, 19; range, 1-39), partial PTSD (median score, 52; range, 40-64), and PTSD scores ≥65.[23]

[b] Low and high combat intensity were Combat Experience Scale scores 5 (25th percentile) and 19 (75th percentile), respectively.

[c] Predicted probability of a continuous outcome CAPS score that falls within defined symptoms ranges for partial PTSD and PTSD.

[d] Of the 16 participants with predeployment PTSD, 8 improved considerably (postdeployment CAPS range, 0-35) and 3 improved slightly (range, 50-78). Symptoms of 3 worsened (range, 78-94); these participants had higher combat intensity (Combat Experience Scale mean score, 22.7 vs 8.4), and 2 of the 3 sustained deployment-related TBI compared with those whose symptoms improved.

health diagnoses, including PTSD, are more frequent in patients with mild TBI.[5] In the present study, however, postdeployment CAPS scores increased with TBI severity. More severe TBI in our participants may reflect more severe physical injury, which has been shown to increase the risk of PTSD.[49] Higher CAPS scores may also reflect nonspecific symptoms that overlap with TBI sequelae. Alternatively, perhaps the overall contexts surrounding severe TBI were more emotionally traumatic than contexts surrounding milder injuries. Although we adjusted for overall combat intensity, that adjustment would not account for the characteristics of any particular traumatic event.

A possible contributor to the overlap of TBI and PTSD symptoms might be that the emotional salience of the event contiguous with TBI may exceed that of the typical civilian or combat-related traumatic event, thereby increasing PTSD risk. Structural and functional brain changes following TBI are likely additional contributors to PTSD outcomes. Prefrontal cortical networks implicated in PTSD[50-52] may be damaged during the course of mild TBI, consequently affecting fear memory processing.[53] Correlations between white matter integrity, cortical function, and postconcussive symptoms provide initial evidence that brain changes associated with mild TBI are distinct from those associated with PTSD or depression.[54-57] Ultimately, high-resolution neuroimaging may help to clarify whether TBI severity reflects neural tissue injury that impedes emotional recovery from stressful events.

There is growing interest in the persistence of postconcussive symptoms and the extensive overlap with anxiety disorders, including PTSD.[58-60] Brain injuries also have been linked to increased suicidality, particularly for individuals with comorbid psychiatric and emotional disturbances, such as PTSD and depression.[61-63] Comorbidity of TBI and PTSD is not unique to deployed service members; motor vehicle accidents and interpersonal assault are 2 common causes of TBI and PTSD in civilians.[64-66] Furthermore, recurrent TBI from contact sports has, as with repeated blast exposure, been linked to greater mental health problems and neurologic abnormalities.[67,68]

Several study limitations should be addressed. As in prior studies,[9,29,69,70] we used retrospective self-report measures, including TBI accounts, which limit causal inference and reflect potentially inconsistent documentation of in-theater events. Furthermore, TBI may be a marker for a traumatic event not otherwise captured by the CES.

In addition, results from the present study may not be generalizable to other populations. Demographic differences between participants and nonparticipants likely reflect the older age and greater military experience of nonparticipants, most of whom were lost to follow-up, possibly resulting from reassignment or discharge. Participation bias likely accounts for mental and physical health differences between participants and nonparticipants. Similar findings have been documented[29] previously and have not been shown to affect study outcomes. Finally, PTSD symptoms were positively skewed, and CAPS threshold scores for partial PTSD and PTSD that were validated in civilians may be conservative for diagnosis in a military population.

Despite these limitations, the present study's prospective design and inclusion of prior psychological and physical trauma are unique contributions to the study of TBI and PTSD. Results suggest that deployment-related TBI may be an important risk factor for PTSD, particularly for individuals with symptoms related to a prior traumatic event.

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Genomic predictors of combat stress vulnerability and resilience in U.S. Marines: A genome-wide association study across multiple ancestries implicates *PRTFDC1* as a potential PTSD gene

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Summary
Background: Research on the etiology of post-traumatic stress disorder (PTSD) has rapidly matured, moving from candidate gene studies to interrogation of the entire human genome in genome-wide association studies (GWAS). Here we present the results of a GWAS performed on samples from combat-exposed U.S. Marines and Sailors from the Marine Resiliency Study (MRS) scheduled for deployment to Iraq and/or Afghanistan. The MRS is a large,

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prospective study with longitudinal follow-up designed to identify risk and resiliency factors for combat-induced stress-related symptoms. Previously implicated PTSD risk loci from the literature and polygenic risk scores across psychiatric disorders were also evaluated in the MRS cohort.
Methods: Participants ($N = 3494$) were assessed using the Clinician-Administered PTSD Scale and diagnosed using the *DSM-IV* diagnostic criterion. Subjects with partial and/or full PTSD diagnosis were called cases, all other subjects were designated controls, and study-wide maximum CAPS scores were used for longitudinal assessments. Genomic DNA was genotyped on the Illumina HumanOmniExpressExome array. Individual genetic ancestry was determined by supervised cluster analysis for subjects of European, African, Hispanic/Native American, and other descent. To test for association of SNPs with PTSD, logistic regressions were performed within each ancestry group and results were combined in meta-analyses. Measures of childhood and adult trauma were included to test for gene-by-environment (GxE) interactions. Polygenic risk scores from the Psychiatric Genomic Consortium were used for major depressive disorder (MDD), bipolar disorder (BPD), and schizophrenia (SCZ).
Results: The array produced >800 K directly genotyped and >21 M imputed markers in 3494 unrelated, trauma-exposed males, of which 940 were diagnosed with partial or full PTSD. The GWAS meta-analysis identified the phosphoribosyl transferase domain containing 1 gene (*PRTFDC1*) as a genome-wide significant PTSD locus (rs6482463; OR = 1.47, SE = 0.06, $p = 2.04 \times 10^{-9}$), with a similar effect across ancestry groups. Association of *PRTFDC1* with PTSD in an independent military cohort showed some evidence for replication. Loci with suggestive evidence of association ($n = 25$ genes, $p < 5 \times 10^{-6}$) further implicated genes related to immune response and the ubiquitin system, but these findings remain to be replicated in larger GWASs. A replication analysis of 25 putative PTSD genes from the literature found nominally significant SNPs for the majority of these genes, but associations did not remain significant after correction for multiple comparison. A cross-disorder analysis of polygenic risk scores from GWASs of BPD, MDD, and SCZ found that PTSD diagnosis was associated with risk sores of BPD, but not with MDD or SCZ.
Conclusions: This first multi-ethnic/racial GWAS of PTSD highlights the potential to increase power through meta-analyses across ancestry groups. We found evidence for *PRTFDC1* as a potential novel PTSD gene, a finding that awaits further replication. Our findings indicate that the genetic architecture of PTSD may be determined by many SNPs with small effects, and overlap with other neuropsychiatric disorders, consistent with current findings from large GWAS of other psychiatric disorders.

1. Introduction

Post-traumatic stress disorder (PTSD) is an anxiety disorder and unique in that exposure to an environmental event (Criterion-A traumatic event; APA, 2000) is a necessary condition for diagnosis. Lifetime prevalence is ~8% in adult Americans (Kessler et al., 1995; Kilpatrick et al., 2013) and is especially high among those exposed to combat, with values ranging from 6% to 31% as reported in a recent review of studies on US combat veterans (Richardson et al., 2010). A large number of demographic and environmental factors and their interactions contribute to PTSD susceptibility, including female gender, age, existence of previous mental health issues, early life stress, as well as severity, duration and number of traumatic incidents, and other factors such as lack of social support (Zoladz and Diamond, 2013). Notably, there are race/ethnic differences in traumatic event exposure, in type of event, age at exposure, as well as the development of PTSD given a specific trauma, with African Americans having somewhat higher risks than whites and Asians (Roberts et al., 2010).

In addition, individual differences in heritable factors affect the risk to develop PTSD. Twin studies indicate that PTSD is moderately heritable, with genetic factors explaining a substantial proportion (30–46%) of vulnerability to PTSD (reviewed e.g. in Wolf et al., 2013). Remaining variance is attributable to the non-shared environment, including trauma encountered during war zone deployments. For some, combat exposure acts as a catalyst that augments the impact of hereditary and environmental contributions to PTSD (Wolf et al., 2013).

A large proportion of the genetic liability for PTSD is also shared with other mental disorders such as anxiety and panic disorder (Goenjian et al., 2008), major depressive disorder (MDD) (Fu et al., 2007; Sartor et al., 2012), and substance use (Xian et al., 2000), hence genes that confer risk for PTSD may also influence risk for other psychiatric disorders and vice versa (Nugent et al., 2008). Such pleiotropic effects have been demonstrated across several psychiatric disorders (Solovieff et al., 2013). For example, a recent study that examined schizophrenia (SCZ), bipolar disorder (BPD), MDD, and attention-deficit/hyperactivity disorder (ADHD) found that SNP-based heritability ranged from 17 to 29% within disorders. Genetic correlations between disorders were also observed with highest associations between SCZ and BPD, and moderate correlations between SCZ and MDD, BPD and MDD, and ADHD and MDD (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Cross-Disorder Group of the Psychiatric Genomics Consortium and Genetic Risk Outcome of Psychosis Consortium, 2013).

Until recently, the genetic contribution to PTSD has been investigated largely via candidate gene association studies (reviewed in Almli et al., 2014; Amstadter et al., 2009; Norrholm and Ressler, 2009). Most research has focused on: (1) the hypothalamic–pituitary–adrenal (HPA) axis, (2) the ascending brainstem locus coeruleus noradrenergic system, and (3) the limbic amygdalar frontal pathway mediating fear processing. Among the over 25 PTSD candidate genes currently reported (Amstadter et al., 2009, 2011; Binder et al., 2008; Boscarino et al., 2011; Cao et al., 2013; Comings et al., 1996; Dragan and Oniszczenko, 2009; Gillespie et al., 2013; Goenjian et al., 2012; Grabe et al., 2009; Guffanti et al., 2013; Hauer et al., 2011; Kolassa et al., 2010; Logue et al., 2013a,b; Lyons et al., 2013; Mellman et al., 2009; Nelson et al., 2009; Ressler et al., 2011; Segman et al., 2002; Solovieff et al., 2014; Voisey et al., 2010; Wilker et al., 2013; Xie et al., 2013), promising findings include associations of PTSD symptoms with the serotonin transporter gene (*SERT*, *SLC6A4*) (Xie et al., 2009), which is linked to depression and anxiety disorders, as well as differential acquisition of conditioned fear and increased amygdala excitability in humans. In addition, *FKBP5*, a co-chaperone of the glucocorticoid receptor involved in the HPA axis, has a significant interaction with severity of child abuse in the prediction of adult PTSD symptoms, indicating a gene by environment (GxE) interaction (Binder et al., 2008). Interestingly, the ankyrin-3 gene (*ANK3*), a known BPD and SCZ gene, was nominally associated with PTSD (Logue et al., 2013b). Although candidate gene studies have not conclusively identified a genetic basis of PTSD, and await replication in independent studies, they suggest a likely polygenic contribution to PTSD development, where a substantial overall genetic effect aggregates over many common variants which individually contribute only minimal effects, further complicated by complex GxE interactions. These findings are in line with the genetic architecture of many psychiatric disorders investigated to date.

To date, only 3 GWASs in PTSD have been published with results implicating several novel loci. The first study on European American (EA) military veterans and their intimate partners identified the retinoid-related orphan receptor alpha (*RORA*) as a potential PTSD gene (Logue et al., 2013a). The second study, including EAs recruited for substance abuse, identified the Tolloid-Like 1 gene (*TLL1*) (Xie et al., 2013), and the third, a study in primarily African American women, implicated a lincRNA (*LINC01090*, alias *AC068718.1*) as a risk factor for PTSD (Guffanti et al., 2013).

In this study we present results from a GWAS on PTSD in the Marine Resiliency Study (MRS), including 3494 trauma-exposed participants. The MRS is a well-characterized, prospective study of Marines and Sailors scheduled for combat deployment to Iraq or Afghanistan, with longitudinal follow-up to track the effect of combat stress (Baker et al., 2012). This young, all-male military cohort is among the largest and most homogenous of PTSD studies available and presents a unique resource to test mechanisms of risk that mediate the link between stressor exposure and outcome, or that moderate or synergize with exposure to mitigate or exacerbate its effect over time. We performed the first GWAS across ancestral groups, including subjects of European, African, Hispanic/Native American, and other ancestries. In addition, we attempted to replicate significant associations in 25 putative PTSD genes from the literature, and tested for main effects and GxE interactions in the MRS. Lastly, we tested for a genetic overlap of PTSD with other psychiatric disorders using polygenic risk profiles from Psychiatric Genomics Consortium (PGC) BPD, MDD, and SCZ GWAS (Purcell et al., 2009).

2. Methods

2.1. Study subjects

Participants were recruited from two studies including military personnel: (1) the Marine Resiliency Study, a prospective PTSD study with longitudinal follow-up (pre- and post-exposure to combat stress) of U.S. Marines bound for deployment to Iraq or Afghanistan (Baker et al., 2012) (here referred to as MRS-I), and (2) the Marine Resiliency Study-II (MRS-II), which followed a very similar protocol. The protocols were approved by the University of California – San Diego Institutional Review Board, and all participants provided written informed consent to participate. Subjects with available genotypes included a total of 3494 unrelated males (MRS-I: $N=2376$; MRS-II: $N=1118$) from 6 different battalions. Based on self-reported race and ethnicity, the cohort was racially 85.5% white and was ethnically 75.5% non-Hispanic. Participant age ranged from 18 to 48 years, with a mean of 23.1 years. Descriptive statistics of the cohort are shown in Table 1.

2.2. Phenotype assessments

Details of phenotype assessments are described in Supplemental methods. In brief, participants were assessed for PTSD diagnosis up to 3 times, once before deployment and 3 and/or 6 month post deployment. Post-traumatic stress (PTS) symptoms were assessed using a structured diagnostic interview, the Clinician Administered PTSD Scale (CAPS), and PTSD diagnosis followed the *DSM-IV* criteria for partial and full PTSD. All participants ($N=3494$) included in this study met the *DSM-IV* criteria A1 event; 38% of them had 2 assessments and 39% had 3 assessments, respectively. For participants assessed at multiple timepoints (i.e. pre- and post-deployments; $N=2689$), the timepoint with the highest CAPS score was used (54% of the CAPS came from pre-deployment, and 46% from post-deployment assessments). Participants meeting criteria for partial or full PTSD diagnosis were designated as cases ($N=940$, including 324 with a full PTSD diagnosis), all other participants were designated controls ($N=2554$). Childhood trauma was assessed in 3385 subjects using a modified version of the Childhood Trauma Questionnaire Short Form (CTQ), and general lifetime trauma was assessed at the time of CAPS assessment in 3494 participants using the Life Events Checklist (LEC), a self-report inventory that inquires about exposure to 16 different potentially traumatic events known to increase risk for PTSD.

Table 1 Descriptive statistics for the Marine Resiliency GWAS cohorts (MRS) studied based on PTSD case versus control status.

	All	MRS-I	MRS-II	PTSD	Controls	p-Value*
Number of Subjects	3494	2376	1118	940	2554	
Age, mean (±SD)	23.1 (3.4)	23.3 (3.5)	22.6 (3.0)	23.0 (3.0)	23.2 (3.5)	0.98
Range	18–48	18–48	18–43	18–38	18–48	
Self reported race						
White	85.5%	84.6%	87.5%	84.1%	86.1%	0.23
African American	4.4%	4.5%	4.1%	4.4%	4.4%	
Other	10.0%	10.8%	8.4%	11.5%	9.5%	
Self reported ethnicity						
Hispanic	24.5%	23.3%	26.2%	25.9%	23.6%	0.16
Non-Hispanic	75.5%	76.7%	73.8%	74.1%	76.4%	
CTQ, mean (±SD)	39.6 (13.5)	40.3 (13.8)	38.0 (12.3)	44.3 (12.8)	37.8 (12.3)	$<2.2 \times 10^{-16}$
Range	25.0–107.5	25.0–106.5	25.0–107.5	25.0–106.5	25.0–107.5	
LEC, mean (±SD)	6.9 (3.5)	6.7 (3.5)	5.8 (3.3)	8.2 (3.4)	5.7 (3.3)	$<2.2 \times 10^{-16}$
Range	0–16	0–16	0–16	0–16	0–16	
Prior deployment	78%	78%	78%	83%	76%	1.4×10^{-5}

* p-Values (PTSD versus Controls) based on Wilcoxon tests (chi-square tests for Race and Ethnicity). CTQ, childhood trauma questionnaire; LEC: life events checklist.

2.3. DNA sample preparation, genotyping, and quality control

Details of sample preparation and genotyping procedures are given in Supplemental methods. In brief, genomic DNA was prepared from blood leukocytes and prepared for genotyping. GWAS-I: genotyping for MRS-I was carried out by Illumina (http://www.illumina.com/) using the HumanOmniExpressExome (HOEE) array with 951,117 loci and resulted in a high initial locus success rate and overall data quality. Additional data cleaning was performed in PLINK v1.07 (Purcell et al., 2007), using standard procedures (Anderson et al., 2010). SNPs were excluded if the call rate was <95%, if they violated Hardy Weinberg Equilibrium ($p < 1 \times 10^{-6}$), or if they showed plate effects (p-value $<1 \times 10^{-8}$ for any one plate or $<1 \times 10^{-4}$ for two or more plates). After removal of problematic DNA samples and markers, the final dataset included 851,541 markers genotyped in 2548 subjects. GWAS-II: a second GWAS for MRS-II samples was carried out by RUCDR (http://www.rucdr.org) using the HOEE array with 967,537 loci and identical data quality procedures were applied. Genotypes ($N = 849{,}099$ SNPs) of 1471 GWAS-II subjects and 23 duplicates (subjects in common with GWAS-I) were then merged with GWAS-I. Array effects were identified by comparing SNP allele frequency variation between GWAS-I and GWAS-II using a chi-squared association test and 132 SNPs with p-values $<5 \times 10^{-8}$ were removed. Reproducibility including 23 replicate pairs (subjects genotyped in both GWAS-I and GWAS-II) was >99.99%. Ancestry was distributed equally across GWAS-I and GWAS-II (chi-squared = 3.50, df = 3, $p > 0.32$; Supplemental Fig. 1), but there were more PTSD cases in GWAS-I compared to GWAS-II (chi-squared = 29.07, df = 1, $p < 6.98 \times 10^{-8}$); a covariate for array was included in the association analyses (see below). The final dataset included 888,113 markers genotyped in 3494 MRS participants (and 525 samples unrelated to this study).

2.4. Genotype imputations

Imputations were performed using the default parameters in IMPUTE2 v2.2.2, using 1000 Genomes Phase 1 integrated variant set haplotypes for the autosomes and the interim set for the X chromosome (see Nievergelt et al., 2014 for details). In brief, prior to imputation, genetic markers that failed Hardy–Weinberg equilibrium ($p < 5 \times 10^{-4}$), or had exceedingly rare alternative alleles (minor allele frequency MAF <0.005) were excluded. Next, genomes were divided into approximately 5 Mb segments, and phasing and imputed genotypes were calculated for each. Imputed markers with low imputation quality values (Info ≤ 0.5) were dropped. A total of 21,692,209 variants were imputed across the two genotyping arrays, resulting in a total of 21,693,469 genotyped and imputed markers for association analyses.

2.5. Ancestry assessment and control for genetic background heterogeneity

Ancestry was determined using genetic information as described in Nievergelt et al. (2013). In brief, genotypes of 1783 ancestry-informative markers (AIMs) were used to determine a subject's ancestry at the continental level for the 7 geographic regions Africa, Middle East, Europe, Central/South Asia, East Asia, Americas, and Oceania. Ancestry estimates were determined using STRUCTURE v2.3.2.1 (Falush et al., 2003) at $K = 7$, including prior population information of the HGDP reference set (Li et al., 2008). To preserve power for the GWAS and reduce type I errors due to population stratification, we aimed to place subjects into large, homogenous groups (European-Americans, EA, $N = 2179$) and groups with simple one-way admixture (African-Americans, AA, $N = 205$; Hispanic and Native Americans, HNA, $N = 640$). All other subjects, including 50 East

Asians, were grouped as Others ($N = 470$) (see Supplemental Fig. 1 for details).

GWAS was performed separately in each of the 4 main ancestral groups. To control for additional genetic background heterogeneity within the 4 ancestral groups, and varying degrees of EA admixture within the HNAs, AAs and others, a principal component analysis (PCA) implemented in EIGENSTRAT (Price et al., 2006) was performed based on 10,000 random, autosomal SNPs separately for each of the 4 groups. Scree plots (data not shown) of the Eigenvalues of the principal components (PC's) indicated that the first five PC's substantially accounted for genetic variability within EA (0.69% cumulative of 5 PC's), AA (6.70% for 5 PCs), HNA (2.81% for 5 PCs), and Others (8.44% for 5 PC's), respectively and were included as covariates in the association analyses.

2.6. Statistical analyses

To test for association of SNPs (at a minor allele frequency MAF > 0.01, $N = 10,446,675$ SNPs) with PTSD status logistic regressions were performed in PLINK for each of the 4 ancestry groups, including battalion, GWAS platform, and the first 5 PC's as covariates. Alleles were coded additively in the GWAS and alternative genetic models were tested post hoc for top hits. To account for uncertainty in SNP imputation, SNP dosages were used rather than allele calls. Resulting p-values were adjusted using genomic control (GC) to correct for genome wide inflation and significance was declared at $p < 5 \times 10^{-8}$. A fixed-effects meta-analysis across ancestry groups was performed based on GC corrected standard errors (SE) using the inverse-variance weighted method in METAL (Willer et al., 2010). Regional association plots were constructed using LocusZoom (Pruim et al., 2010), using the 1000 Genomes project Europeans as reference population and R^2 as the measure for linkage disequilibrium (LD).

Candidate gene analyses: associations for single gene analyses selected from the literature are reported at a nominal p-value of 0.05. Gene-wide significance was estimated using the set-based permutations in PLINK with default parameters. Gene by environment (GxE) interactions were calculated using a robust SE method (Voorman et al., 2011) as implemented in the R-package rms (Harrell, 2014).

Polygenic risk score analyses: risk score analyses were performed in EA MRS participants based on data downloaded from the PGC website for bipolar disorder (BPD), major depressive disorder (MDD), and Schizophrenia (SCZ). LD-pruned SNP sets for the 3 disorders were filtered at varying p-value thresholds (P_T) (at $p < 0.01$, <0.05, <0.10, <0.20, <0.30, <0.40, and <0.50). A risk score for each MRS participant was computed by the number of risk alleles weighted by the log of the odds ratios (ORs). To test if the polygenic risk scores for these disorders could predict PTSD status in MRS, logistic regressions with the specific SNP sets were performed, including battalion, GWAS platform, and the first 5 PC's as covariates.

Power calculations for the association analysis were performed using the case–control module for discrete traits (Purcell et al., 2003) at $D' = 1$ and parameters derived from the MRS.

2.7. VA replication sample

GWAS hits in the discovery sample were tested for replication in an independent cohort including 491 VA samples. Sample ascertainment, characterization, genotyping, and data cleaning methods used have been described elsewhere in detail (Logue et al., 2013a). Briefly, the sample is a subset of a cohort of military veterans and their intimate partners ascertained from two studies performed at U.S. Department of Veterans Affairs (VA) medical centers. All participants were assessed using the CAPs with excellent inter-rater reliability (kappa = 0.87). Genotyping was performed using the Illumina HumanOmni2.5-8 array and samples were excluded if they had a call rate of <95% or if their reported sex did not match their inferred sex based on X-chromosome genotypes. Only white non-Hispanic subjects (based on a STRUCTURE (Falush et al., 2003; Pritchard et al., 2000) analysis of 10,000 markers) with a *DSM-IV* defined PTSD Criterion-A traumatic event were included in the analysis. The sample analyzed includes 491 white non-Hispanic veterans and their intimate partners including 313 lifetime-PTSD cases and 178 trauma-exposed controls. Association between the SNP and lifetime PTSD was tested using PLINK (v. 1.07). First, the sample was analyzed using a logistic model adjusting for the top 3 PC's computed in EIGENSTRAT based on 10,000 randomly chosen markers.

3. Results

Meta-analysis of GWASs with PTSD in subjects of European (EA), African (AA), Hispanic/Native American (HNA), and other descents. Genome-wide association studies for PTSD were performed with genotypes of 2179 EA's, 640 HNA's, 205 AA's, and 470 subjects of other or mixed ancestral descent. The genomic control (GC) inflation factor lambda was close to 1.0 in all analyses (see Supplemental Fig. 2 for QQ-plots). GC-corrected p-values were combined in a meta-analysis and resulted in a genome-wide significant association for a SNP in the phosphoribosyl transferase domain containing 1 gene (rs6482463 in *PRTFDC1*; OR = 1.47, SE = 0.06, $p = 2.04 \times 10^{-9}$) (Fig. 1A and Supplemental Table 1A). *PRTFDC1* is a 104 kb long gene on chromosome 10, including 9 exons. The top SNP rs6482463 (imputed based on the genotyped proxy SNP rs6482463, $R^2 = 0.995$, imputation info score = 0.99) is located in a ~40 kb LD-block spanning most of intron 3 (Fig. 1B). An analysis of the large EA subgroup identified a different SNP (rs2148269, imputed) as top hit in this gene. SNP rs2148269 is located in the same LD-block as rs6482463 ($R^2 = 0.27$) (see Supplemental Fig. 3A and B for the EA Manhattan and regional association plots). However, the meta-analysis top SNP rs6482463 shows consistent odds ratios (OR) across all 4 ancestry groups, and a test for heterogeneity between studies was not significant ($p = 0.9$ for Cochran's Q; Table 2A). Given the parameters from the meta-analysis of rs6482463 (MAF = 0.27, relative risk = 1.324), a power calculation indicated that the study was sufficiently powered (~80%) to detect an effect size of this magnitude (OR = 1.47).

Replication of the *PRTFDC1* association with PTSD was attempted in an independent military cohort (VA replication

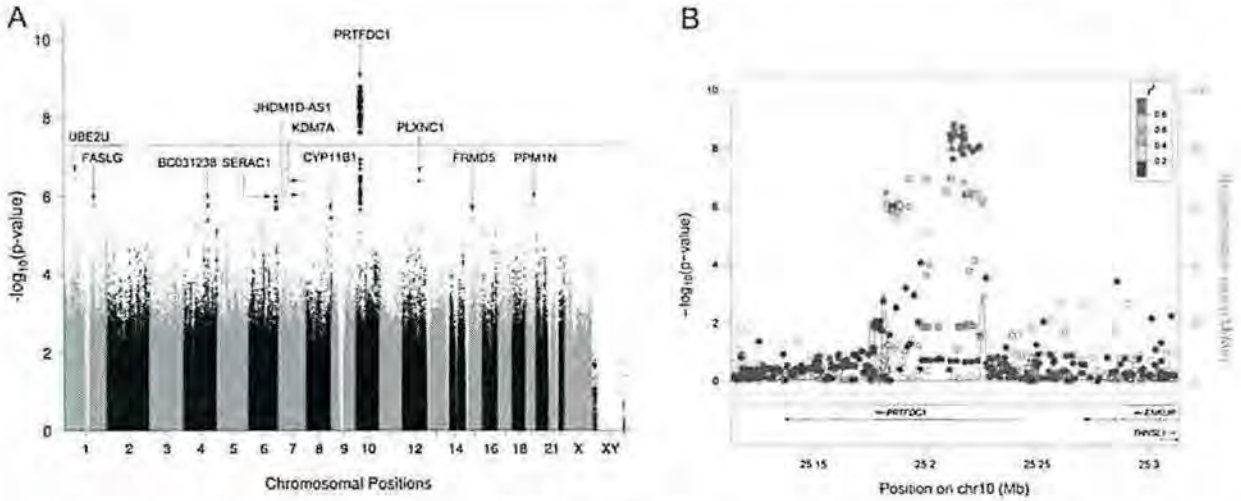

Figure 1 (A) Manhattan plot of genome-wide association results for PTSD from a meta-analysis of subjects from mixed ancestries. The red line represents genome-wide significance at $p < 5 \times 10^{-8}$ and the dashed line represents suggestive evidence for association at $p < 5 \times 10^{-6}$. (B) Regional association plot, showing significant regions in *PRTFDC1* on chromosome 10. Results are reported for the most significant SNP rs6482463 from the meta-analysis. The color of each circle is based on R^2 with rs6482463 and recombination rates are based on European reference subjects from the 1000 Genomes Project.

sample). The imputed SNP rs6482463 was not available, but rs1033962 (a SNP 3678 bp apart) was genotyped in both MRS and NCPTS. Associations for rs1033962 in the MRS meta-analysis were slightly less strong than for the top SNP rs6482463 ($p = 4.93 \times 10^{-9}$; Table 2B). Association of rs1033962 in the smaller NCPTS replication study was not significant ($N = 491$; $p = 0.14$). However, the direction of the effect of the A allele was consistent with MRS, and a meta-analysis of MRS and NCPTS showed no heterogeneity (Cochran $p = 0.91$) and further decreased the p-value to 2.06×10^{-9}.

We also explored alternative statistical models for *PRTFDC1* associations with PTSD, extending from the basic model with an additively coded SNP effect, and the covariates battalion, GWAS platform, and 5 PC's for population stratification. Compared to the additive model ($p = 2.04 \times 10^{-9}$), recessive and dominant genetic models did not show stronger effects for rs6482463 ($p = 2.03 \times 10^{-3}$ or $p = 3.2 \times 10^{-9}$, respectively). In addition, we tested the effects of age, different types of traumas (CTQ, LEC, and prior deployments; see Table 1), and GxE interactions on PTSD status (Supplemental Table 1B). Age and the 3 types

Table 2 Meta-analyses of *PRTFDC1* associations with PTSD for (A) the most significant imputed SNP rs6482463 in four Marine Resiliency Study (MRS) ancestry groups, and (B) for the genotyped SNP rs1033962 in MRS and an independent replication sample from the National Center for PTSD/Boston (NCPTS).

Study	Ancestry	A1	A2	MAF	N subjects	OR	SE	P	Q
(A) Association analysis for rs6482463									
MRS	EA	A	G	0.22	2179	1.41	0.08	2.98×10^{-05}	
	AA	A	G	0.46	205	1.49	0.26	0.118	
	HNA	A	G	0.31	640	1.58	0.14	1.25×10^{-03}	
	OTH	A	G	0.31	470	1.55	0.18	0.012	
Meta		**A**	**G**	–	**3494**	**1.47**	**0.06**	$\mathbf{2.04 \times 10^{-09}}$	**0.90**
(B) Association analysis for rs1033962									
MRS	EA	A	G	0.22	2179	1.40	0.08	4.48×10^{-05}	
	AA	A	G	0.47	205	1.45	0.25	0.148	
	HNA	A	G	0.31	640	1.57	0.14	1.37×10^{-03}	
	OTH	A	G	0.31	470	1.52	0.17	0.016	
Meta	**All**	**A**	**G**	–	**3494**	**1.45**	**0.06**	$\mathbf{4.93 \times 10^{-09}}$	**0.90**
NCPTS	EA	A	G	0.21	491	1.28	0.17	0.144	
Meta	**All**	**A**	**G**	–	**3985**	**1.43**	**0.06**	$\mathbf{2.06 \times 10^{-09}}$	**0.91**

MAF, minor allele frequency for A1 allele; OR, odds ratio; SE, standard error of the mean; *Q*, *p*-value for Cochran's *Q* statistic; meta, inverse-variance weighted meta-analysis; EA, European American; AA, African American; HNA, Hispanic and Native American descent; OTH, other.

of trauma significantly predicted PTSD in univariate analyses ($p < 0.05$ in all cases), and explained between 2.8% (age) and 14.9% (LEC) of the variability (% VE). Adding these predictors to the top SNP rs6482463 slightly decreased the p-values for the SNP effect for models including SNP plus age, CTQ, or prior deployment, respectively. Tests for GxE interactions using the LEC, CTQ or prior deployment were not significant ($p > 0.05$ in all cases). Finally, a cumulative model including SNP, age, LEC, CTQ and prior deployment (plus the standard covariates battalion, GWAS platform, and 5 PC's for ancestry) was most significant in predicting PTSD status ($p = 4.07 \times 10^{-94}$) and explained ~20% of the variance (Supplemental Table 1B).

In addition to the genome-wide significant association with *PRTFDC1*, SNPs in 26 genes met the threshold for suggestive evidence of association ($p < 5 \times 10^{-6}$), including SNPs in 10 genes from the meta-analysis (Fig. 1A) and 15 genes in specific ancestry groups. A summary for the top SNPs per gene are shown in Table 3 (see also extended data in Supplemental Table 2). As expected based on the size of the subsets, most of the associations meeting suggestive evidence were found in the largest EA subgroup (see also Manhattan plot for EA in Supplemental Fig. 2). There was considerable heterogeneity across the 4 ancestral groups in regards to the effect of the top SNPs. The direction of the effects across the 26 genes was consistent only for 7 of the top SNPs, and Cochran's Q value showed significant heterogeneity across studies for 12 associations, including all 4 of the SNPs meeting suggestive evidence in the AAs and both SNPs meeting suggestive evidence in the HNA's. No SNP met suggestive evidence for association in the 470 subjects of 'other' descent.

In addition to testing for a main SNP effect on PTSD diagnosis we also tested for an interaction of childhood trauma (CTQ) and the top SNPs listed in Table 3 (GxE interaction). Six SNPs showed nominally significant GxE interactions in one or more ancestry groups. However, none of them remained significant after correction for multiple comparisons (at a threshold of $p < 0.002$ for 26 tests performed).

3.1. Comparison of PTSD genes reported in the literature with results from the MRS GWAS

We compared the results from the MRS association analyses in the EA and AA subgroups for 25 genes with significant association with PTSD for either a main SNP effect and/or a significant GxE interaction previously reported in the literature (see Table 4 for EA and Supplemental Table 3 for AA). Most of the genes were identified in candidate gene studies, but *LINC01090* (Guffanti et al., 2013), *RORA* (Logue et al., 2013a), and *TLL1* (Xie et al., 2013) came from recent GWASs, thus meeting the stricter level for genome-wide significance in the original studies. We first investigated the specific SNP reported in the literature and found that none of the reported SNPs were nominally significant in the MRS EA or AA subgroups. Next we tested all available SNPs within the 25 genes for association with PTSD. The number of SNPs per gene available in the MRS GWAS ranged from 11 SNPs in *RGS2* to 1976 SNPs in *ANK3*. With the exception of *APOE*, all genes included

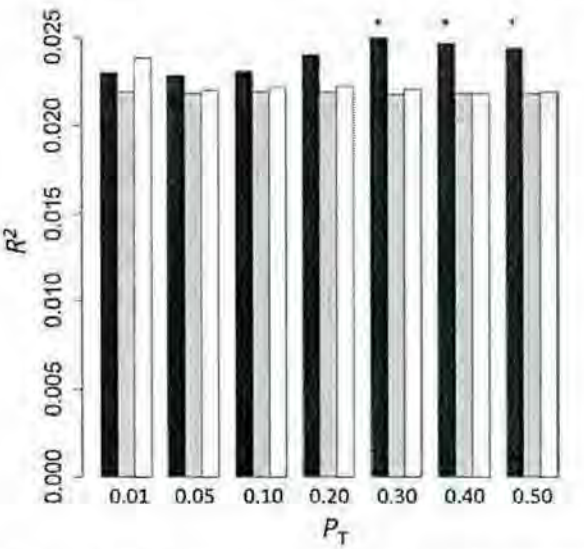

Figure 2 Polygenic risk score profiling in European American subjects, using discovery sets from GWAS on bipolar disorder (BPD, black bars), major depressive disorder (MDD, gray bars), and schizophrenia (SCZ, white bars) from the Psychiatric Genomic Consortium (PGC). The x-axis shows results at seven p-value thresholds ($P_T = 0.01, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50$). The y-axis shows the Nagelkerke pseudo R^2, the proportion of variance in PTSD case–control status explained by the risk score profile. * indicates nominal significance at $p < 0.05$.

at least one nominally significant SNP in the EA and/or AA subgroup. However, after controlling for multiple comparisons at the gene level (not yet considering the number of genes tested), none of these associations remained significant.

Significant GxE interactions were reported for SNPs in 7 genes (Table 4), predominantly including childhood trauma as the environmental factor. We did not replicate a GxE effect in these 7 genes in the MRS ($p > 0.05$). GxE interactions in 4 other genes without a reported GxE in the original studies were nominally significant in MRS, but did not meet the threshold after correction for multiple comparisons ($p < 0.002$ for 25 genes tested).

3.2. Association of cross-disorder polygenic risk scores in the MRS PTSD GWAS

We also tested for a genetic overlap of PTSD with bipolar disorder (BPD), major depressive disorder (MDD), and schizophrenia (SCZ) using polygenic risk scores. These scores, an aggregate of many SNPs with small individual effects retrieved from large PGC GWAS studies, were used at different p-value thresholds (P_T), ranging from 0.01 to 0.5 (Fig. 2). We found that the polygenic risk scores for BPD explained a significant proportion of phenotypic variance in the MRS for $P_T = 0.3$ (Nagelkerke $R^2 = 0.025$, $p = 0.028$), $P_T = 0.4$ ($R^2 = 0.025$, $p = 0.037$), and $P_T = 0.5$ ($R^2 = 0.024$, $p = 0.047$). Polygenic risk scores from GWAS of SCZ and MDD did not significantly predict PTSD in the MRS.

Table 3 Top hits from genome-wide association studies with PTSD in subjects of European (EA), African (AA), Hispanic/Native American (HNA), and other descents, and meta-analysis across ancestry groups.

SNP	CHR	Gene	Location	Allele 1/2	EA		AA		HNA		Other		Meta-analysis			
					P_{main}	P_{GxE}	P_{main}	P_{GxE}	P_{main}	P_{GxE}	P_{main}	P_{GxE}	Q	OR	P_{main}	Direction
rs138384996	1	*UBE2U*	Downstream	G/A	1.6E−05	0.08	0.686	0.999	0.11	0.17	0.022	0.16	0.82	0.24	**4.1E−07**	−+−−
rs4916008*	1	*JAK1*	Intron	C/T	**4.0E−06**	1.00	0.49	0.26	0.58	0.34	0.90	0.06	0.033	1.40	2.6E−04	+++−
rs74939664	1	*LPHN2*	Downstream	T/C	**2.0E−06**	0.33	0.66	0.05	0.43	0.81	0.27	0.13	0.06	1.73	3.5E−05	++−+
rs2312236*	1	*POGK*	Upstream	T/C	0.99	0.47	0.94	0.30	**2.9E−06**	0.93	0.11	0.62	**4.4E−05**	1.13	0.204	+−+−
rs6681010	1	*FASLG*	Downstream	G/A	0.001	0.66	0.010	0.06	0.19	0.74	0.08	0.27	0.83	0.46	**2.0E−06**	−−−−
rs4511180	1	*PTPRV*	Exon	A/G	0.16	0.37	**7.7E−07**	0.81	0.65	0.98	0.30	0.80	**2.8E−05**	1.12	0.049	+++−
rs3100127	1	*LGR6*	Upstream	C/A	0.14	0.26	**1.3E−06**	0.58	0.55	0.91	0.19	0.81	**2.5E−05**	0.89	0.05	−−−+
rs10737854	1	*RGS7*	Intron	G/A	**4.6E−06**	0.53	0.93	0.96	0.83	0.11	0.036	0.82	0.07	0.78	1.7E−05	−++−
rs187093517	2	*UBE2E3*	Upstream	A/G	**2.8E−07**	0.61	0.26	0.72	0.84	0.29	0.77	0.73	**0.038**	0.51	1.1E−05	−−++
rs62275374	4	*LRPAP1*	Upstream	G/A	**4.5E−06**	**0.006**	0.88	0.76	0.13	0.27	0.62	0.95	**0.004**	0.73	1.3E−03	−−++
rs1380630	4	*BC031238*	Upstream	T/C	1.8E−05	0.54	1.00	0.55	0.21	0.14	0.029	0.49	0.56	0.69	**1.9E−06**	−+−−
rs10457838	6	*UST*	Intron	C/T	0.004	0.65	**2.2E−06**	0.83	0.84	1.00	0.27	0.53	**4.3E−06**	0.88	0.039	−+−−
rs115028822	6	*SERAC1*	Intron	C/A	1.6E−04	0.44	0.09	0.42	0.08	0.09	0.08	0.23	0.99	0.45	**1.6E−06**	−−−−
rs79485117	7	*KDM7A*	Intron	C/T	1.5E−05	0.92	0.36	**0.027**	0.14	0.94	0.036	0.19	0.89	0.58	**4.4E−07**	−−−−
rs2471320*	7	*JHDM1D-AS1*	Downstream	T/C	2.0E−05	0.69	0.80	**0.003**	0.22	0.18	0.19	0.16	0.96	0.55	**4.2E−06**	−−−−
rs2616978	8	*CSMD1*	Intron	T/C	0.12	0.11	0.014	**0.050**	**2.8E−06**	0.77	0.40	0.87	**1.0E−06**	1.04	0.46	−++−
rs142570922	8	*CYP11B1*	Upstream	A/C	2.2E−04	0.27	0.82	0.82	0.003	**0.036**	0.15	**0.027**	0.40	1.34	**3.8E−06**	+−++
rs10511822	9	*LINGO2*	Intron	C/T	**3.1E−06**	0.15	0.80	0.77	0.79	0.94	0.73	0.38	0.05	0.73	1.6E−04	−−−+
rs58649573	9	*LHX2*	Downstream	T/C	0.37	0.70	**9.0E−07**	0.25	0.18	0.06	0.94	0.77	**8.4E−06**	1.04	0.48	−+++
rs2148269	10	*PRTFDC1*	Intron	A/G	**4.6E−06**	0.90	0.93	0.66	0.007	0.74	0.653	0.36	0.25	0.69	**8.0E−07**	−−−−
rs6482463	10	*PRTFDC1*	Intron	G/A	3.0E−05	0.98	0.12	0.73	0.001	0.91	0.012	0.30	0.90	0.68	**2.0E−09**	−−−−
rs73220799	12	*PLXNC1*	Upstream	C/T	**4.3E−06**	0.91	0.046	0.09	0.47	0.05	0.12	0.38	0.48	1.96	**4.4E−07**	++++
rs9545302*	13	*LINC01080*	Downstream	A/C	**1.4E−06**	0.88	0.22	0.92	0.66	0.46	0.40	0.79	**6.3E−04**	0.84	0.004	−+++
rs78826942	15	*FRMD5*	Intron	T/C	3.1E−05	0.18	0.14	0.34	0.024	0.69	0.60	0.18	0.32	0.52	**3.8E−06**	−+−−
rs148952004	19	*PPM1N*	Intron	A/G	**3.8E−06**	0.87	0.38	0.57	0.10	0.21	0.75	0.020	0.56	0.23	**1.8E−06**	−−−−
rs199563271	20	*PTPRT*	Intron	CACAT/C	**1.4E−06**	0.24	0.65	**0.020**	0.82	0.24	0.85	0.59	0.028	0.47	1.4E−04	−+−−
rs6528940	X	*MAGEC1*	Downstream	T/C	**1.3E−06**	0.09	0.28	0.68	0.033	0.23	0.44	0.49	**1.1E−04**	1.13	0.003	+−−+

Gene by environment (GxE) analyses are based on Childhood trauma. *p*-Values for the main analysis (P_{main}) in bold meet suggestive ($p < 5 \times 10^{-06}$) or genome-wide significance ($p < 5 \times 10^{-08}$). GxE interaction *p*-values (P_{GxE}) and Q values (*p*-value for Cochran's *Q* statistic) in bold meet nominal significance ($p < 0.05$). * Genotyped SNPs, all other SNPs listed are imputed.

Table 4 PTSD association analysis of SNPs in 25 putative PTSD genes from published PTSD candidate gene and genome-wide association studies in 2179 MRS subjects of European descent.

Gene	Reported in literature						MRS GWAS				
	Study	Ancestry	Reported SNP	P/P_{GxE}	P	P_{GxE}	N SNPs	Top SNP	P	P_{GxE}	P_{gene}
ADCYAP1R1 (PAC1)	Ressler et al. (2011)	AA	rs2267735	Y/–	0.66	0.25	65	rs6968349	**0.017**	0.84	0.47
ANK3	Logue et al. (2013a,b)	EA	rs9804190	Y/N	0.93	0.76	1976	rs139604943	**0.008**	0.85	0.53
APOE	Lyons et al. (2013)	EA	rs429358, rs7412[a]	Y/Y	0.96	0.27	27	rs1081105	0.05	**0.036**	1
CHRNA5	Boscarino et al. (2011)	EU	rs16969968	Y/Y	0.22	0.67	72	rs518425	0.07	0.61	1
COMT	Kolassa et al. (2010)	AA	rs4680	N/Y	0.53	0.55	116	rs174686	**0.049**	0.64	1
CRHR1	Amstadter et al. (2011)	Other	rs12944712	Y/–	0.41	0.52	1034	rs116897693	**0.023**	0.63	0.24
DRD2	Comings et al. (1996)	Other	rs1800497	Y/–	0.06	0.93	177	rs75924850	**0.021**	0.48	0.74
DRD4	Dragan and Oniszczenko (2009)	EU	VNTR	Y/N	NA	NA	14	rs4987059	0.10	0.53	1
DTNBP1	Voisey et al. (2010)	EA	rs9370822	Y/–	0.90	**0.004**	346	rs116647843	0.06	0.82	0.86
FKBP5	Binder et al. (2008)	AA	rs9296158	N/Y	0.15	0.32	239	rs9366890	**0.015**	0.23	0.19
GABRA2	Nelson et al. (2009)	Other	rs279836	N/Y	0.36	0.13	255	rs148139959	**0.024**	0.16	1
HTR2A	Mellman et al. (2009)	AA	rs6311	Y/N	0.88	0.17	232	rs6314	**0.049**	0.20	0.79
LINC01090 (AC068718.1)[c]	Guffanti et al. (2013)	AA, EA	rs10170218	Y/–	1.00	0.93	1582	rs6759539	**0.002**	0.28	0.27
NR3C1	Hauer et al. (2011)	EA	rs41423247	Y/–	0.73	0.86	162	rs79590198	0.09	**0.022**	1
RGS2	Amstadter et al. (2009)	EA	rs4606	N/Y	0.49	0.45	11	rs141129523	**0.022**	0.23	0.08
RORA[c]	Logue et al. (2013a)	EA, AA	rs8042149	Y/N	0.99	0.71	1706	rs12442490	**0.003**	0.65	0.58
SLC18A2	Solovieff et al. (2014)	EA, AA	rs363276	Y/–	0.16	0.52	107	rs363238	**0.022**	0.34	0.48
SLC6A3 (DAT1)	Segman et al. (2002)	EA	VNTR	Y/–	NA	NA	86	rs144782362	**0.008**	0.37	0.33
SLC6A4 (SERT)	Grabe et al. (2009)	EU	rs4795541, rs25531[b]	Y/Y	NA	NA	43	rs28914827	0.14	0.54	1
SRD5A2	Gillespie et al. (2013)	AA	rs523349	Y/N	0.11	0.69	137	rs77929608	**0.049**	0.39	0.65
STMN1	Cao et al. (2013)	Other	rs182455	Y/–	0.51	0.66	32	rs4659395	0.09	0.23	1
TLL1[c]	Xie et al. (2013)	EA, AA	rs6812849	Y/–	0.46	0.24	434	rs113712660	**0.017**	0.19	0.62
TPH1	Goenjian et al. (2012)	EA	rs2108977	Y/–	0.67	0.58	42	rs544437	0.25	0.75	1
TPH2	Goenjian et al. (2012)	EA	rs11178997	Y/–	0.46	0.32	250	rs183063707	**0.017**	0.13	0.73
WWC1 (KIBRA)	Wilker et al. (2013)	AA	rs10038727	Y/N	0.99	0.44	469	rs17551315	**0.001**	0.14	0.10

Gene by environment (GxE) analyses are based on Childhood trauma. Nominally significant p-values ($p < 0.05$) are bolded and gene-set p-values are corrected for the number of SNPs tested per gene. P/P_{GxE}: indicates if the study reported a significant main effect (P: yes/no) or a significant gene by environment interaction (P_{GxE}: yes/no). P_{gene}: set-based empirical p-values for each gene, corrected for the number of SNPs (N SNPs) tested per gene. Top SNP: best result for the tested MRS SNPs.

[a] rs429358, rs7412 ($\varepsilon 2$, $\varepsilon 3$, $\varepsilon 4$).

[b] rs4795541 (L/S) + rs25531 (L_A/L_G)

[c] Genome-wide significant genes from published GWA studies.

4. Discussion

We present the first multi-ethnic GWAS of PTSD to date, including subjects of European, African, Native American/Hispanic, and other ancestry, typically found in U.S. military cohorts. Participants were recruited from the MRS, a large, prospectively assessed cohort of Marines and Sailors with index deployments to Iraq or Afghanistan (Baker et al., 2012). This all-male study included 3494 subjects exposed to a *DSM-IV* criteria A1 traumatic event and represents one of the largest and most homogenous PTSD GWAS to date. Due to the military culture and training of the participants we did not require the endorsement of the A2 criteria i.e. that the traumatic experience is accompanied by intense fear, helplessness, or horror. However, removal of A2 from the *DSM-IV* criterion set does not seem to substantially increase the number of people who qualify for PTSD diagnosis (Karam et al., 2010), and A2 has been dropped entirely in the new *DSM-V* PTSD definition.

The GWAS meta-analysis across ancestry groups identified the phosphoribosyl transferase domain containing 1 gene (*PRTFDC1*) as a potential PTSD gene meeting genome-wide significance. This finding was supported by a smaller, independent VA cohort including 491 EA veterans and their intimate partners with 313 lifetime-PTSD cases (Logue et al., 2013a). *PRTFDC1* is a ~100 kb long gene located on chromosome 10p12. It encodes the phosphoribosyltransferase domain-containing protein 1, a relatively small protein with highest expression in the brain. PRTFDC1 belongs to the purine/pyrimidine phosphoribosyltransferase family and is a paralog of HPRT1, but may have lost its ancestral HPRT activity (Keebaugh et al., 2007). However, *PRTFDC1* has been reported as a possible tumor-suppressor gene that is frequently silenced by aberrant promoter hypermethylation (Suzuki et al., 2007). To our knowledge *PRTFDC1* has not yet been implicated in GWAS of PTSD or other psychiatric disorders and its potential role in the etiology of PTSD remains to be determined.

As expected from a meta-analysis across ancestries, the *PRTFDC1* top hit from the meta-analysis was a SNP with a similar effect across multiple ancestry groups. This SNP (rs6482463) is located in a ~40 kb LD block spanning most of intron 3. The GWAS for the largest subgroup, including 2179 EAs, identified a different top hit in the same LD block, complicating a functional analysis of these findings. However, based on the UCSD genome browser annotations the whole region of the LD block shows enrichment in H3K27Ac and H3K4Me3 histone marks, indicative of high transcriptional activity (see Supplemental Fig. 4).

A hallmark of PTSD association studies are frequent findings of GxE interactions, where the effect of a gene on PTSD risk is exaggerated in the presence of a high trauma burden (Koenen et al., 2008). For example, this has been found for childhood trauma (Binder et al., 2008) as well as adult trauma such as combat exposure (Lyons et al., 2013). The thoroughly characterized MRS includes pre- and post-combat exposure trauma assessments, allowing for detailed testing of GxE interactions. We found that, while the overall model to predict PTSD status improved when we included trauma exposure into the model (from a model with baseline covariates and the SNP alone explaining ~4% of the variability to the complete model including trauma exposure explaining a cumulative ~20% of the variability), GxE interactions for childhood trauma, adult life events, or previous combat deployments were not significant. Since our cohorts experienced a relatively large trauma burden, with significantly more trauma of all types reported by participants diagnosed with PTSD compared to Marines with low PTS symptoms (see Table 1), we conclude that power in MRS was similar to other studies that reported significant interactions. However, GxE interactions have been difficult to replicate and have a high potential to be false positives (Duncan and Keller, 2011). Recent methods based on model-robust estimates of standard errors are promising, especially in the context of genome-wide GxE analyses (Voorman et al., 2011).

In addition to the genome-wide significant *PRTFDC1* we found SNPs in 25 genes with suggestive evidence for association with PTSD. These results stem from specific ancestry groups and/or from the meta-analysis across groups. A comparison of findings between the different ancestry groups is limited by the much smaller size of the non-EA subgroups. Interesting genes with suggestive evidence for association include *CSMD1*, a gene previously implicated in large GWAS of other psychiatric disorders (Schizophrenia Psychiatric Genome-Wide Association Study, 2011), genes (*JAK1*, *FASLG*) related to immune response, a pathway that has previously been implicated for PTSD by GWAS (Guffanti et al., 2013) as well as gene expression studies (Glatt et al., 2013), and genes (*UBE2E3*, *UBE2U*) from the ubiquitin system, which has been implicated in the etiology of schizophrenia and bipolar disorder (Bousman et al., 2010). Before conclusions can be drawn however these genes must be replicated in larger GWASs and meta-analyses currently planned by the PGC PTSD working group (Koenen et al., 2013).

We also compiled a list of genes that have been reported in the literature to be significantly associated with PTSD, either showing a main effect for the genetic marker, and/or a significant GxE interaction (Amstadter et al., 2009, 2011; Binder et al., 2008; Boscarino et al., 2011; Cao et al., 2013; Comings et al., 1996; Dragan and Oniszczenko, 2009; Gillespie et al., 2013; Goenjian et al., 2012; Grabe et al., 2009; Guffanti et al., 2013; Hauer et al., 2011; Kolassa et al., 2010; Logue et al., 2013a,b; Lyons et al., 2013; Mellman et al., 2009; Nelson et al., 2009; Ressler et al., 2011; Segman et al., 2002; Solovieff et al., 2014; Voisey et al., 2010; Wilker et al., 2013; Xie et al., 2013). Since most studies were performed in subjects of either European or African descent, we used these specific ancestry groups for comparison with MRS. We found that most of the 25 candidate genes showed nominally significant associations in MRS for at least one of the SNPs tested. However, none of these results remained significant after appropriate Bonferroni corrections. Comparing the number of PTSD cases and overall study sizes between MRS and other studies indicated that we were adequately powered to detect many of the reported effects at least for the EA studies. A similar, well-powered study recently failed to replicate findings for 20 PTSD candidate genes after appropriate adjusting for multiple testing (Solovieff et al., 2014). This lack of replication may be due to a relatively large heterogeneity between PTSD studies, which are complicated by the requirement of exposure to a traumatic event, leading to potential differences in type, timing of, and time since trauma, and the observed GxE interactions.

However, it has been demonstrated that reports from candidate gene association studies (Sullivan, 2007), and especially GxE interactions (Duncan and Keller, 2011), have a high false discovery rate and a robust replication of findings is now a policy required by many journals.

In regards to our inability to replicate previous findings from GWASs, which met the stringent genome-wide significant thresholds, power calculations indicated that MRS was sufficiently powered for a replication of rs8042149 in *RORA* (Logue et al., 2013a) for EA's (OR 2.1 in original study and 1.22 in MRS; data not shown). However, the association of rs6812849 in *TLL1* (Xie et al., 2013) was originally detected in a larger study, and rs10170218 in *LINC01090* (Guffanti et al., 2013) was originally found in an all-female AA cohort, which was also larger than the all-male MRS AA cohort, and MRS findings for these genes remain inconclusive.

On the other hand, the large MRS GWAS was able to replicate a recent finding from a candidate gene study including 300 genes (Solovieff et al., 2014) that demonstrated for the first time the existence of common SNPs between PTSD severity and bipolar disorder based on cross-disorder polygenic risk score analyses. We used the standard polygenic scoring approach (Purcell et al., 2009) with results from the PGC for MDD, BP, and SCZ (Cross-Disorder Group of the Psychiatric Genomics Consortium and Genetic Risk Outcome of Psychosis Consortium, 2013) and found that PTSD diagnosis was predicted by risk scores derived from BPD, but not from MDD or SCZ. Our results for BPD reached significance at p-value thresholds >0.3 from the original GWAS, similar to the PTSD candidate gene study (Solovieff et al., 2014). Pleiotropic effects across a range of psychiatric disorders have recently been reported (Cross-Disorder Group and Genetic Risk Outcome, 2013) and provide exciting new insights into the genetic architecture of PTSD and other psychopathologies.

Power analyses for the population-based MRS cohort GWAS indicated increased power using a broad definition for PTSD, including 616 subjects with partial, and 324 subjects with a full *DSM-IV* based diagnosis (data not shown), compared to confining the sample to subjects with full PTSD diagnosis only. For example, the smaller size of the full PTSD case group would diminish the significance of our top finding for rs6482463 in *PRTFDC1* (OR = 1.47, SE = 0.06, $p = 2.04 \times 10^{-9}$) to below genome-wide significance, despite similar effect size (OR = 1.46, SE = 0.096, $p = 7.64 \times 10^{-5}$). As an alternative to using a specific disease cut-off we have considered quantitative analyses of PTSD symptoms. However, population-based studies require careful consideration of PTSD symptom distributions (e.g. CAPS symptoms in MRS are best characterized by a zero-inflated negative binomial distribution; Yurgil et al., 2014), which may lead to increased rates of false positives if not modeled appropriately. The broad PTSD definition used in this study may potentially limit a direct comparison with findings from other PTSD studies. In addition, our findings stem from a very homogenous all-male military cohort and generalizability into other population groups may be limited.

In summary, this first multi-ethnic PTSD GWAS highlights the potential to increase power of GWAS through meta-analyses of multi-ethnic association analyses for SNPs with consistent effects across ancestries. We found evidence for *PRTFDC1* as a novel PTSD gene, a finding that awaits further replication. And lastly, the genetic architecture of PTSD may be determined by many SNPs with small effects, and overlap with other neuropsychiatric disorders, consistent with current findings from large GWAS of other psychiatric disorders, suggesting that genetic contributions to psychiatric disorders may not completely map to present diagnostic categories (Cross-Disorder Group of the Psychiatric Genomics Consortium and Genetic Risk Outcome of Psychosis Consortium, 2013).

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2014.10.017.

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Prospective Associations Between Traumatic Brain Injury and Postdeployment Tinnitus in Active-Duty Marines

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Objective: To examine whether cause, severity, and frequency of traumatic brain injury (TBI) increase risk of postdeployment tinnitus when accounting for comorbid posttraumatic stress disorder. **Design:** Self-report and clinical assessments were done before and after an "index" deployment to Iraq or Afghanistan. **Setting, Participants, and Measures:** Assessments took place on Marine Corps bases in southern California and the VA San Diego Medical Center. Participants were 1647 active-duty enlisted Marine and Navy servicemen who completed pre- and postdeployment assessments of the Marine Resiliency Study. The main outcome was the presence of tinnitus at 3 months postdeployment. **Results:** Predeployment TBI increased the likelihood of new-onset postdeployment tinnitus (odds ratio [OR] = 1.86; 95% confidence interval [CI], 1.28-2.70). Deployment-related TBIs increased the likelihood of postdeployment tinnitus (OR = 2.65; 95% CI, 1.19-5.89). Likelihood of new-onset postdeployment tinnitus was highest for those who were blast-exposed (OR = 2.93; 95% CI, 1.82-6.17), who reported moderate-severe TBI symptoms (OR = 2.22; 95% CI, 1.22-3.40), and who sustained multiple TBIs across study visits (OR = 2.27; 95% CI, 1.44-4.24). Posttraumatic stress disorder had no effect on tinnitus outcome. **Conclusions:** Participants who were blast-exposed, sustained multiple TBIs, and reported moderate-severe TBI symptoms were most at risk for new-onset tinnitus. **Key words:** *blast, combat, military, posttraumatic stress disorder, PTSD, TBI, tinnitus, traumatic brain injury*

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TINNITUS, defined as the perception of sound in the absence of an external auditory source,[1] is the number one service-related Veterans Affairs (VA) disability.[2] Tinnitus and hearing loss combined cost more than $1 billion annually in disability benefits, excluding treatment and hearing aid expenditures.[2] In 2012, roughly a quarter of VA beneficiaries received disability payments for tinnitus including 115 638 new cases, an increase of 12% over the previous year.[2] This 12% yearly rise has been consistent since the early 2000s. In addition, 1% to 3% of patients with tinnitus experience long-term health consequences, including sleep disturbance,[3] depression,[4,5] anxiety,[6-10] somatoform disorders,[11] and suicide.[12,13]

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Although typically associated with hearing loss, tinnitus may occur in the absence of hearing difficulty.[14] In the general population, 20.7% of those with high exposure to noise complain of tinnitus compared with 7.5% of adults with little or no noise exposure.[15] In the US military population, more than 60% report tinnitus several months following a blast event.[16] Contact with detonations caused by improvised explosive devices has been one of the leading causes of traumatic brain injury (TBI) in the Iraq and Afghanistan battle zones.[17-19] Rates of blast-related hearing loss and tinnitus have risen significantly since the onset of the war in Iraq.[20] Patients with blast injuries are at least 2.5 times more likely to sustain tinnitus than those with a TBI from nondetonation incidents,[21] and at least 60% to 75% of veterans with a history of mild TBI report tinnitus.[22] Roughly 1.4 million civilians sustain TBI per year in the United States,[23] and a separate survey from Oregon noted that 5% of those with tinnitus list an explosion as the proximate cause of tinnitus.[24] Thus, it may be prudent to screen for tinnitus among US civilians as well as military personnel.

Although the intracranial mechanism of blast-related tinnitus is unclear, the initial cochlear injury may be traced to a generalized central neural syndrome. The cochlea is uniquely vulnerable to primary blast injury since the air-liquid interface of the round window can be subject to direct overpressure through the exquisitely thin and elastic tympanic membrane. In contrast, the brain is somewhat protected by absorption of the pressure wave by the skull. The initial shock wave from a blast leads to shearing of tissues due to differential pressures acting on liquid versus more rigid structures such as blood vessels.[25] This shearing force directly injures the brain and cochlea, causing an inflammatory response, oxidative stress–induced neural degeneration,[26] and subsequent neural alteration both within the cochlea and its auditory pathway.[27]

Establishing a direct, causal link between blast exposure and tinnitus has been limited by the retrospective, cross-sectional nature of available accounts[28-30] and the existence of comorbid psychiatric disorders such as posttraumatic stress disorder (PTSD).[31,32] Failure to differentiate tinnitus symptoms from these comorbidities further hinders the identification of tinnitus-specific treatment modalities. This prospective study examines the effects of blast-related TBI and injury severity on tinnitus while accounting for comorbid and preexisting symptoms, including PTSD symptoms, prior TBI, and tinnitus.

METHODS

Approval for human participants was obtained from University of California San Diego, VA San Diego Research Service, and Naval Health Research Center (VA R&D and UCSD institutional review board approval #070533). All participants gave written informed consent before participation.[33]

Study design and participants

Participants were a subset of the 2600 active-duty Marine and Navy servicemen enrolled in the Marine Resiliency Study (MRS),[33] a prospective, longitudinal investigation of 4 infantry battalions stationed in southern California. Servicemen were deployed to Iraq or Afghanistan between July 2008 and May 2012 for approximately 7 months (the "index deployment") and were assessed approximately 1 month before deployment, 1 week postdeployment (only self-report questionnaires), and 3 and 6 months postdeployment. Data collected at 6 months postdeployment were not analyzed here because of reduced follow-up rates and insufficient number of symptom cases. A priori exclusions were 34 participants without an index deployment and 66 officers who were significantly older ($P < .001$) and had lower combat experience scores ($P < .001$) than enlisted participants. Of the remaining 2500, 1829 completed the 3-month postdeployment assessment and were eligible for analysis.

Data from these remaining participants were examined for any hearing difficulty at 3 months postdeployment. Tones of 500, 1000, 3000, and 6000 Hz were presented at 35 dB (Grayson Stadler Audiometer, Eden Prairie, Minnesota). This screening test was performed to ensure participants would be able to hear and understand study assessments. Preliminary analyses showed that the 6000-Hz frequency was most commonly missed; however, χ^2 tests revealed no difference in rates of tinnitus for this group compared with those who missed other frequencies. To ensure our sample included only those with serviceable hearing within *conversational frequency range*, we excluded 116 participants who failed to hear frequencies at or below 3000 Hz at 3 months postdeployment. Of the remaining 1713, 66 were missing relevant data and were excluded from analysis. The final sample for this study included 1647 participants.

Measures

Complete MRS methodology has been reported previously.[33] Descriptions of measures relevant to this study follow. Demographic information (age, ethnicity, race, battalion) was collected via self-report surveys before deployment and was included in analysis as potential covariates.[34,35]

Presence of tinnitus was assessed before deployment and 3 months postdeployment with a single "yes/no" item on an interview-assisted questionnaire, "Do you

have ringing in the ears?" Participants who responded "yes" as having ringing in the ears at the time of assessment were categorized as having tinnitus. Participants were also asked whether or not they had an ear infection at the time of assessment. To account for any influence on tinnitus outcome, the presence of an ear infection at the 3-month postdeployment assessment was tested for any significant univariate associations with postdeployment tinnitus.

Head injury events were assessed via interview before deployment and 3 months postdeployment. Interviewers gathered details of each reported injury, including injury cause or mechanism and symptom severity. Traumatic brain injury was defined as any head injury that resulted in loss of consciousness or altered mental status (ie, dazed, confused, or seeing stars, and/or posttraumatic amnesia).[36–38] Mild TBI was any TBI resulting in a loss of consciousness of less than 30 minutes and posttraumatic amnesia for less than 24 hours.[39] Because the time between predeployment and postdeployment assessments was broader than the duration of the deployment, nondeployment TBIs sustained between assessment visits ($n = 34$) were included in analyses to account for potential effects on tinnitus.[40,41] As these were a small minority, for succinct communication, all TBIs sustained between predeployment and 3-month postdeployment assessments are labeled "deployment-related" for this article.

Posttraumatic stress symptoms were assessed before deployment and 3 months postdeployment using the Clinician-Administered PTSD Scale[42] in accordance with symptom criteria from the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition, Text Revision).[43] PTSD/partial PTSD group classification required exposure to a traumatic event (ie, actual or threatened death or serious injury, or threat to physical integrity to self or others) but did not require a response of extreme fear, helplessness, or horror.[44,45] In addition, PTSD classification required at least 1 reexperiencing symptom, 3 avoidance symptoms, and 2 hyperarousal symptoms; partial PTSD classification required at least 1 reexperiencing symptom and either 3 avoidance symptoms or 2 hyperarousal symptoms.[46] Symptoms must have occurred at least once within the past month (frequency ≥ 1), causing at least moderate distress (intensity ≥ 2).[47] Participants with partial PTSD and PTSD were evaluated together ($n = 200$ at predeployment; $n = 341$ at postdeployment) to examine the effects of clinically significant symptoms on tinnitus.

A modified 16-item version of the Combat Experiences Scale from the Deployment Risk and Resilience Inventory[48,49] was used to assess combat intensity 1 week after deployment. Item responses were measured on a 5-point Likert scale, ranging from 0 (never) to 4 (daily or almost daily). Total scores ranged from 0 to 64, with higher scores indicating greater combat intensity.

Analysis

Continuous predictors were centered prior to analysis. A priori analysis of variance and χ^2 tests revealed battalion differences in predeployment demographic and psychological characteristics, shown in Table 1. Thus, we included battalion as a covariate to correct for these and any other unknown battalion differences such as training schedules, battalion leadership and cohesion, and timing of study assessments. Categorical demographic predictors were dummy-coded with the following reference groups: battalion 1, white, and non-Hispanic. Reference groups for categorical diagnostic predictors were participants with no prior tinnitus, no PTSD, and no TBI.

Presence of tinnitus at 3 months postdeployment was the dependent variable for all analyses. Predictor variable selection was conducted via univariate logistic regression analysis of each predictor variable.[50] Variables with $P < .2$ associations were included as predictors in the full multivariate analysis. The multivariate analysis tested all main effects and all 2-way interactions between clinical diagnostic and combat exposure variables. Sensitivity analyses tested effects of TBI characteristics, including injury mechanism (blast vs nonblast), severity (mild vs moderate/severe), and frequency (single vs multiple). Significance levels for 3 sensitivity analyses were Bonferroni adjusted with an α level of .017. All data analyses were performed using Statistical Package for Social Sciences (SPSS; version 21.0).[51]

RESULTS

Sample characteristics

Battalion differences in demographic and psychosocial variables have been published previously.[33] Mean (SD) age of participants was 22.4 (3.36) years. Roughly 84.7% of participants were white, 4.5% were African American, and 10.9% were of mixed or other racial descent. The majority (78.5%) was non-Hispanic. Approximately 74.3% were junior enlisted (E1-E3), and 44.6% were deployed prior to the index deployment. Mean (SD) combat intensity score was 13.0 (11.1).

Of the 1647 participants, 219 (13.2%) had tinnitus before the index deployment and 250 (15.1%) had tinnitus after deployment. Of the 250 participants with postdeployment tinnitus, 141 (56.4%) had new-onset tinnitus and 109 (43.6%) had tinnitus both before and after the index deployment. Observed prevalence of deployment-related TBI was 34.8% for those with new-onset postdeployment tinnitus compared with 17.4% for those with no pre- or postdeployment tinnitus

TABLE 1 *Battalion differences in predeployment characteristics*

	Battalion 1 ($n = 232$)	Battalion 2 ($n = 469$)	Battalion 3 ($n = 501$)	Battalion 4 ($n = 445$)
Predeployment characteristic[a]				
Age, mean (SD), y	21.4 (2.6)	22.1 (3.5)	22.9 (3.3)	22.8 (3.5)
% Non-Hispanic	78	81.4	73.3	76.3
% White	84.5	87.4	83.4	83.2
% Rank E1-E3	81.5	81.8	73.6	62.5
% Prior deployed	50.4	43.9	42.9	43.6
% TBI	62.9	60.3	55.9	48.5
% Tinnitus	3.9	16.2	22.0	5.4
Assessment scores, mean (SD)				
CAPS	15.8 (14.8)	15.0 (13.6)	14.7 (15.3)	13.6 (14.6)
Childhood trauma	40.0 (13.0)	38.9 (12.5)	38.4 (12.0)	42.1 (14.8)
SF-12 Physical Health	54.6 (5.6)	53.7 (6.8)	54.2 (6.0)	53.7 (6.2)
SF-12 Mental Health	49.2 (8.4)	48.9 (9.4)	49.9 (8.4)	50.5 (8.1)

Abbreviations: CAPS, Clinician-Administered PTSD Scale; E1-E3, junior enlisted; PTSD, posttraumatic stress disorder; SF-12, 12-item Short Form Health Survey; TBI, traumatic brain injury.
[a]Small but significant differences in age ($F_3 = 13.5$; $P < .001$; $\eta p^2 = 0.02$), ethnicity ($\chi^2_3 = 9.4$; $P < .05$; $\varphi = 0.08$), rank ($\chi^2_3 = 52.1$; $P < .001$; $\varphi = 0.12$), predeployment TBI ($\chi^2_3 = 18.2$; $P < .001$; $\varphi = 0.10$), predeployment tinnitus ($\chi^2_3 = 78.0$; $P < .001$; $\varphi = 0.22$), childhood trauma score ($F_3 = 7.4$; $P < .001$; $\eta p^2 = 0.01$), and SF-12 Physical Health score ($F_3 = 1.5$; $P < .01$; $\eta p^2 = 0.003$) and Mental Health score ($F_3 = 2.9$; $P < .05$; $\eta p^2 = 0.005$). There were no significant battalion differences for the current sample in race, prior deployments, or predeployment CAPS total symptom score.

($\chi^2_1 = 24.7$; $P < .0001$; $\varphi = 0.13$). Before deployment, 195 (11.8%) had partial PTSD or PTSD and 907 (55.1%) had previously sustained TBI. After predeployment, 336 (20.4%) had partial PTSD or PTSD at their 3-month postdeployment assessment and 316 (19.2%) sustained deployment-related TBI. Prevalence of TBI-related characteristics before and after the index deployment is shown in Table 2. Of the 1015 participants who reported TBI at either assessment visit, 825 (81.3%) had mild TBI, 648 (63.8%) sustained injuries from nonblast events, and 415 (40.9%) sustained only 1 TBI across assessment visits.

Univariate predictor selection

Univariate test results are shown in Table 3. Postdeployment tinnitus was significantly associated with battalion membership ($P < .01$), and those with tinnitus were more likely to be non-Hispanic (81.2% vs 76.4%) and white (88.6% vs 84%) than those without tinnitus. Participants with postdeployment tinnitus were more likely to have had prior tinnitus (43.6% vs 7.9%), prior TBI (63.6% vs 54.8%), and prior partial PTSD or PTSD (9.6% vs 5.4%). Those with postdeployment tinnitus also had higher combat intensity scores (mean [SD] = 15.9 [12.7] vs 12.5 [10.7]) and had higher rates of

TABLE 2 *Rates of TBI reported pre- and postdeployment*

TBI characteristic	Predeployment ($n = 907$)	Postdeployment[a] ($n = 316$)	Total[b] ($N = 1015$)
% Mechanism			
Nonblast	82.4	20.9	63.8
Blast	17.6	79.1	36.2
% Severity[c]			
Mild	82.2	88.0	81.3
Moderate/severe	13.8	11.1	15.5
% Frequency			
Single	44.7	66.8	40.9
Multiple	55.3	33.2	59.1

Abbreviation: TBI, traumatic brain injury.
[a]Postdeployment reports of TBI include all deployment-related TBIs ($n = 282$) and nondeployment TBIs sustained between pre- and postdeployment assessments ($n = 34$). There were no significant differences between deployment and nondeployment TBIs; thus, nondeployment TBIs were included in the analysis to account for any potential effects on tinnitus.
[b]Total number of participants with TBI characteristic across pre- and postdeployment visits.
[c]Percentages may not sum to 100% due to missing data.

TABLE 3 *Variable selection via univariate logistic regression*

Variable	3 mo postdeployment		
	No tinnitus ($n = 1397$)	Tinnitus ($n = 250$)	*P*
Demographic			
Age, mean (SD), y	22.5 (3.4)	22.3 (3.1)	.620
% Battalion[a]			.002
Battalion 1	15.1	8.4	
Battalion 2	27.0	36.8	
Battalion 3	31.1	26.8	
Battalion 4	26.8	28.0	
% Non-Hispanic	76.4	81.2	.095
% White[b]	84.0	88.6	.064
% Rank E1-E3	74.3	72.8	.618
Predeployment			
% Tinnitus	7.9	43.6	.000
% Partial PTSD or PTSD[c]	5.4	9.6	.070
% History of TBI	54.8	63.6	.010
Deployment			
Combat intensity,[d] mean (SD)	12.5 (10.7)	15.9 (12.7)	.000
% TBI	17.5	31.6	.000
Postdeployment			
% Partial PTSD or PTSD[e]	18.6	32.4	.000
% Ear infection	1.4	1.2	.774

Abbreviations: E1-E3, junior enlisted; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a]Cohort sizes are 232 in battalion 1, 469 in battalion 2, 501 in battalion 3, and 445 in battalion 4. Demographic and psychiatric differences across battalions have been published previously.[33]

[b]African Americans constituted roughly 5.0% of participants with no postdeployment tinnitus, 1.6% of those with postdeployment tinnitus, and 4.5% of all participants.

[c]Of the participants *without* postdeployment tinnitus, approximately 7.5% had partial PTSD before deployment and 4.2% had PTSD. Of the participants *with* postdeployment tinnitus, 9.6% had partial PTSD before deployment and 6.0% had PTSD.

[d]Mean (SD) combat intensity score across all participants in this sample was 13.0 (11.1).

[e]Of the participants *without* postdeployment tinnitus, approximately 13.2% had partial PTSD after deployment and 5.4% had PTSD. Of the participants *with* postdeployment tinnitus, 22.8% had partial PTSD after deployment and 9.6% had PTSD.

deployment TBI (31.6% vs 17.5%) and postdeployment partial PTSD or PTSD (32.4% vs 18.6%). Participants with and without postdeployment tinnitus did not differ as a function of age, rank, or ear infection.

Multivariate analysis

Variables with univariate associations with postdeployment tinnitus ($P < .2$) were selected for the multivariate model. Demographic variables were battalion, ethnicity, and race; clinical diagnostic and deployment-related variables were prior tinnitus, prior and deployment-related TBI, combat intensity, and prior and postdeployment partial PTSD/PTSD.

Results of the multivariate model are shown in Table 4. There was a significant association between battalion and postdeployment tinnitus ($P < .01$), with battalion 2 increasing the likelihood of postdeployment tinnitus by a factor of 2.01 ($P < .02$) compared with battalion 1. Prior tinnitus and prior TBI independently increased the likelihood of postdeployment tinnitus by factors of 27.44 ($P < .001$) and 1.86 ($P < .02$), respectively, and showed significant interaction ($P < .01$). Post hoc comparisons revealed that prior TBI significantly increased the likelihood of postdeployment tinnitus for those *without* prior tinnitus (odds ratio = 1.86; 95% confidence interval [CI], 1.28-2.70) but not for those *with* prior tinnitus (odds ratio = 0.59; 95% CI, 0.34-1.02). Deployment-related TBIs increased the likelihood of postdeployment tinnitus by a factor of 2.65 ($P < .02$). As expected, there was no significant interaction between deployment-related TBI and prior tinnitus.

Neither combat intensity nor partial PTSD/PTSD was significantly associated with postdeployment tinnitus. The nonsignificant effect of PTSD was confirmed via 2 post hoc analyses that tested (1) the combined effects of pre- and postdeployment partial PTSD/PTSD as a single diagnostic predictor, and (2) the effects of pre- and postdeployment PTSD, excluding participants with partial PTSD. Furthermore, 2 additional post hoc analyses (3) including participants with any hearing difficulty within the 500- to 3000-Hz range ($n = 116$), and (4) excluding

Table 4 *Multivariate logistic regression predicting postdeployment tinnitus*

Variable	Coef.	SE	*P*	OR	95% CI for OR
Intercept	−2.96	0.33			
Battalion, *main effect*			.000		
Battalion 2	0.70	0.29	.014	2.01	1.15-3.51
Battalion 3	−0.48	0.31	.129	0.62	0.34-1.15
Battalion 4	0.07	0.33	.838	1.07	0.56-2.04
Ethnicity[a]	−0.40	0.21	.051	0.67	0.39-1.14
Race[b]	−0.12	0.24	.609	0.89	0.48-1.63
Predeployment partial PTSD	0.76	0.51	.138	2.13	0.78-5.77
Predeployment tinnitus	3.31	0.32	.000	27.44	14.55-51.75
Predeployment TBI	0.62	0.25	.013	1.86	1.14-3.04
Predeployment TBI × predeployment tinnitus	−1.15	0.37	.002	0.32	0.15-0.65
TBI without predeployment tinnitus	0.62	0.19	.001	1.86	1.28-2.70
TBI with predeployment tinnitus	−0.53	0.28	.057	0.59	0.34-1.02
Combat intensity, *centered*	0.01	0.02	.424	1.01	0.98-1.05
Deployment TBI[c]	0.97	0.41	.017	2.65	1.19-5.89
Postdeployment partial PTSD	0.44	0.41	.285	1.55	0.70-3.44

Abbreviations: CI, confidence interval; OR, odds ratio; PTSD, posttraumatic stress disorder; SE, standard error; TBI, traumatic brain injury.

[a]Results are reported for Hispanics compared with non-Hispanics (reference group).

[b]Results are reported for non-whites compared with whites (reference group).

[c]There were no significant differences between deployment and nondeployment TBIs sustained between pre- and postdeployment assessments ($n = 35$). Nondeployment TBIs were included in the analysis to account for any potential effects on tinnitus.[40,41]

participants with any hearing difficulty within the full 500- to 6000-Hz range ($n = 209$) did not alter model outcomes.

Sensitivity analyses

Table 5 shows results of sensitivity analyses of TBI mechanism (blast vs nonblast), severity (mild vs moderate/severe), and frequency (single vs multiple) on the likelihood of postdeployment tinnitus. Variables for TBI were collapsed across pre- and postdeployment because the small number of nonblast and moderate-severe TBIs caused problems with model convergence.

There was a main effect of TBI mechanism on postdeployment tinnitus ($P < .01$) as well as an interaction with prior tinnitus ($P < .01$). For those with no prior tinnitus, nonblast and blast TBIs significantly increased the likelihood of postdeployment tinnitus by factors of 1.91 (95% CI, 1.20-3.32) and 2.93 (95% CI, 1.82-6.17), respectively. For those with prior tinnitus, TBI mechanism had no effect on postdeployment tinnitus.

There was a significant interaction between TBI severity and prior tinnitus ($P < .01$). For those with no prior tinnitus, mild and moderate/severe TBIs significantly increased the likelihood of postdeployment tinnitus by factors of 1.99 (95% CI, 1.29-3.62) and 2.22 (95% CI, 1.22-3.40), respectively. For those with prior tinnitus, TBI severity had no effect on postdeployment tinnitus.

Finally, there was a main effect of TBI frequency ($P < .02$) and a significant interaction between frequency and prior tinnitus ($P < .01$). For those with no prior tinnitus, a single TBI increased the likelihood of tinnitus outcome by a factor of 1.79 (95% CI, 1.09-2.97) and multiple TBIs increased the likelihood by a factor of 2.27 (95% CI, 1.44-4.24). For those with prior tinnitus, TBI frequency had no effect on postdeployment tinnitus.

DISCUSSION

In our model, prior tinnitus and TBI were each independently associated with postdeployment tinnitus. Prevalence of tinnitus was 13.2% before deployment and 15.1% after deployment, with 8.6% new-onset postdeployment tinnitus. Rates of pre- and postdeployment tinnitus are consistent with prior reports of a prevalence of 15.6% in soldiers deployed to Iraq.[52] Prior tinnitus occurred in roughly 43.6% of participants with postdeployment tinnitus. Interestingly, not all those with prior tinnitus sustained the symptom postdeployment. Of the 219 participants with prior tinnitus, 110 (50.2%) were asymptomatic after the index deployment. Of these, 67.3% sustained TBI prior to the index deployment. Tinnitus for these participants may be an acute symptom from prior TBIs that diminished over time. In addition, those who were asymptomatic after deployment had lower rates of deployment-related TBI (19.1% vs 27.5%) and lower mean combat intensity (11.5 vs 14.5) than

TABLE 5 *Sensitivity analyses of TBI characteristics[a] on postdeployment tinnitus*

TBI characteristic	Prior tinnitus	Coef.	SE	OR	95% CI for OR
Mechanism					
Nonblast	No	0.65	0.24	1.91	1.20-3.32
Nonblast	Yes	−0.88	0.33	0.42	0.22-1.24
Blast	No	1.07	0.24	2.93	1.82-6.17
Blast	Yes	−0.09	0.35	0.92	0.46-1.59
Severity					
Mild	No	0.69	0.22	1.99	1.29-3.62
Mild	Yes	−0.61	0.30	0.54	0.30-1.35
Moderate/severe	No	0.80	0.30	2.22	1.22-3.40
Moderate/severe	Yes	−0.26	0.50	0.77	0.29-1.34
Frequency					
Single	No	0.58	0.25	1.79	1.09-2.97
Single	Yes	−0.97	0.39	0.38	0.18-1.20
Multiple	No	0.82	0.23	2.27	1.44-4.24
Multiple	Yes	−0.46	0.31	0.63	0.34-1.41

Abbreviations: CI, confidence interval; OR, odds ratio; SE, standard error; TBI, traumatic brain injury.
[a]For all sensitivity analyses, TBIs were collapsed across pre- and postdeployment visits because of the small number of deployment-related nonblast TBIs and moderate/severe TBIs causing problems with model convergence.

those with both pre- and postdeployment tinnitus. Alternatively, some participants may have had intermittent tinnitus that was not present after the index deployment.

Traumatic brain injury sustained before the index deployment increased the likelihood of new-onset postdeployment tinnitus, suggesting that a history of TBI may be a risk factor for tinnitus for those with no prior symptoms. As 44.6% of our participants were deployed prior to their index deployment, tinnitus and TBI symptoms reported at the predeployment assessment may be attributable to prior deployments. Independent of any prior tinnitus, those with deployment-related TBI were 2.7 times as likely to report tinnitus after deployment compared with those with no TBI. Furthermore, prevalence of deployment-related TBI was significantly higher for those with new-onset postdeployment tinnitus than those with no pre- or postdeployment tinnitus. These findings are consistent with those of previous cross-sectional studies that show associations between TBI and tinnitus and/or hearing difficulty.[29,30,53,54]

Tinnitus was associated with TBI characteristics. Consistent with prior cross-sectional studies showing higher rates of tinnitus[20] and hearing problems[28] following blast versus nonblast injuries, postdeployment tinnitus was nearly twice as likely for those with nonblast TBI and nearly 3 times as likely for those with blast TBI compared with those with no TBI. In addition, tinnitus was 1.8 times as likely after a single TBI and 2.3 times as likely after multiple TBIs compared with tinnitus occurrence in those with no TBI. Furthermore, new-onset postdeployment tinnitus was 1.9 times as likely for those with mild TBI and 2.2 times as likely for those with moderate/severe TBI. These results suggest a dose-response relationship between TBI characteristics and tinnitus such that more numerous and more severe injuries increase the risk of tinnitus.

This study found no associations between tinnitus and PTSD or combat intensity; thus, associations of TBI with tinnitus cannot be attributed to psychiatric symptoms or other environmental factors. These results are contrary to previous findings that suggest that tinnitus may be associated with exposure to harsh sounds from firearms, artillery, and mechanized equipment during deployment,[16] as well as long-term stress including emotional exhaustion,[55] fatigue,[56] and PTSD.[57] In one study,[58] 75% of participants with PTSD had tinnitus whereas only 15.9% of those without PTSD reported tinnitus. However, this was a retrospective study and there was a large cultural overlay in which tinnitus was thought to indicate "soul loss." Causes of the onset of tinnitus, such as head trauma, noise-induced hearing loss, or prior ear infections, were not addressed in that study.[58] Nevertheless, neural pathways damaged in TBI-related tinnitus may differ from those impacted by psychological stress. Emotional or psychological distress associated with tinnitus has been shown to activate a neural network involving the anterior cingulate cortex, insula, hypothalamus, and amygdala.[59] This same network has been implicated in other perceptual disorders such as phantom limb pain and may reflect the nonspecific influence of psychological distress.[59] Further investigation is needed to determine whether neural networks associated with stress-related tinnitus are distinct from TBI-induced tinnitus.

Neural changes following cochlear trauma have been demonstrated using acoustically evoked discharge, otoacoustic emissions, protein expression, and neuroimaging.[27,60-65] The initial shock wave from a blast leads to shearing of tissues,[25] directly injuring the cochlea and leading to an inflammatory response with subsequent neural degeneration.[26] Animal models of TBI demonstrate loss of ribbon synapses from inner hair cells to the auditory nerve in mild cases and then deterioration of outer hair cells of the cochlea, leading to altered auditory nerve activity.[66] Upregulation of BDNF (brain-derived nerve growth factor), a modulator of neuronal plasticity, is noted in spiral ganglion neurons and intracranially, and the spontaneous discharge rate of auditory fibers increases as a result of acoustic trauma.[62] These changes accompany enhanced subcortical disinhibition in the brainstem and inferior colliculus.[67] Disinhibition and prolonged excitation occur along the tonotopic map of the auditory cortex immediately following a loud sound.[68] Neural activity of the central auditory system, including reorganization of the cortical tonotopic map, is associated with an imbalance between excitation and inhibition in the auditory pathway.[64] These studies suggest immediate changes in expression of excitatory and inhibitory neurotransmitters and increased spontaneous signal transmission to the dorsal cochlear nucleus in the brainstem. Along with multiple biomarkers of neural plasticity in the cochlea and auditory tract and nuclei, there is a reorganization of frequency representation in the dorsal cochlear nuclei and inferior colliculus and a long-term change in the temporal pattern of neural activity. In animal studies, these neural alterations continue for at least 1 month following acute noise injury.[27,67]

In addition, functional magnetic resonance imaging and positron emission tomography studies show that tinnitus is associated with increased activity in the frontal lobe, limbic system, and auditory association cortex and show asymmetry in the primary auditory cortex and metabolic asymmetry between hemispheres.[64] Magnetoencephalography, which measures spatial and temporal neural activity, has identified activity between the anterior cingulum and right frontal cortex correlating with tinnitus distress,[69] although it is unclear whether differences in patterns are more related to hearing loss or tinnitus.[70] Future studies should address the specific pathophysiology of TBI-induced tinnitus to ascertain any differences from noise-induced injury.

Several study limitations warrant consideration. Self-reported symptoms, including reports of TBI, PTSD, and tinnitus, are subject to bias and misclassification errors, thus limiting causal inference. Our tinnitus measurement did not capture symptom severity, duration, or functional impact, all of which may have important clinical implications[24] and should be explored in future studies. Although it was made clear to participants that their individual responses and data would be kept confidential and would not be reported to their command, participants may still have had concerns regarding the impact of reporting PTSD and tinnitus symptoms on their careers or future disabilities compensation. It should be noted that information obtained via self-report and interview was not relevant for research study compensation. A post hoc analysis that excluded those with partial PTSD did not alter study findings; therefore, it is unlikely that the inclusion of partial PTSD diluted any potential effects of PTSD on tinnitus.

In addition, our hearing evaluation was not intended to detect hearing loss above 6000 Hz but ensured that participants had normal hearing within conversational frequency range (500-3000 Hz at 35 dB). A more thorough audiometric examination was not possible due to ethical constraints. Finally, our data are from an all-male cohort of military service members, many of whom experienced repeated blast exposure; thus, results may not be generalizable to civilian populations, although they are likely generalizable to other military groups.

Despite these limitations, our prospective, longitudinal data suggest that TBI may be a significant risk factor for new-onset tinnitus. Furthermore, risk of tinnitus is higher for blast TBIs than for nonblast TBIs and increases with injury severity and frequency. Our findings provide support for the use of TBI assessments as potential screening tools for tinnitus, particularly for those exposed to explosive devices. Blast head trauma may be a different clinical entity than tinnitus from blunt head trauma and should be treated differently. In the closely related vestibular system, military service members with blast head injury demonstrated longer latency times on motor control testing than those with mild TBI post–blunt head trauma. Blast exposure appears to produce a more global injury pattern, whereas closed blunt head injury in the mouse model shows more focal brain injury.[71,72]

Notably, this study did not find an association between PTSD and tinnitus. Traumatic brain injury–induced tinnitus in this population may be a nonsomatoform diagnosis with distinct pathophysiology and should be addressed by referral from primary care early in the treatment of TBI. Early treatment may influence the neural alterations noted in cochlear and cranial studies immediately following injury. Although treatment modalities are beyond the scope of this article, both medications and cognitive therapy have shown promise in taking advantage of neuroplasticity to "redirect" neural circuits during the repair phase after injury.[27,66] Imaging studies that measure spatial and temporal neural activity may lead to a better understanding and ultimately treatment of this ubiquitous symptom.

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Psychophysiology in the Study of Psychological Trauma: Where Are We Now and Where Do We Need to Be?

D.T. Acheson, M.A. Geyer and V.B. Risbrough

Abstract Posttraumatic stress disorder (PTSD) is a major public health concern, which has been seeing increased recent attention partly due to the wars in Iraq and Afghanistan. Historically, research attempting to understand the etiology and treatment of PTSD has made frequent use of psychophysiological measures of arousal as they provide a number of advantages in providing objective, non-self-report outcomes that are closely related to proposed neurobiological mechanisms and provide opportunity for cross-species translation. Further, the ongoing shift in classification of psychiatric illness based on symptom clusters to specific biological, physiological, and behavioral constructs, as outlined in the US National Institute of Mental Health (NIMH) Research Domain Criteria project (RDoC), promises that psychophysiological research will continue to play a prominent role in research on trauma-related illnesses. This review focuses on the current state of the knowledge regarding psychophysiological measures and PTSD with a focus on physiological markers associated with current PTSD symptoms, as well as markers of constructs thought to be relevant to PTSD symptomatology (safety signal learning, fear extinction), and psychophysiological markers of risk for developing PTSD following trauma. Future directions and issues for the psychophysiological study of trauma including traumatic brain injury (TBI), treatment outcome studies, and new wearable physiological monitoring technologies are also discussed.

Keywords Psychophysiology · PTSD · Startle · Heart rate variability · Electrodermal response · TBI

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Contents

1 Introduction

Posttraumatic stress disorder (PTSD) is a major public health concern with lifetime prevalence rates in the USA estimated to be 6.8–12.2 %, and 12-month prevalence rates estimated to be 3.5 % (Breslau 2009). Due to the wars in Iraq and Afghanistan, PTSD has received significant attention in the past 10–13 years, in terms of both popular media coverage and funds directed toward its research. This attention is warranted, given that rates of PTSD have increased in service members by 656 % since 2001 and the cost to the US Department of Defense (DoD) for treating these service members doubled between 2007 and 2012 (Blakeley and Jansen 2013 Congressional Research Service Report). In addition, it is important to note that PTSD affects more than just combat veterans and occurs in civilians following physical and sexual assaults, forced captivity, muggings/robberies, motor vehicle accidents, natural disaster, and life-threatening illness among other events (Breslau 2009). The DSM-IV classification of PTSD consisted in exposure to the traumatic event, as well as 3 clusters of symptoms: re-experiencing, avoidance and numbing, and hyperarousal. With the recent publication of DSM-5, the definition has expanded into 4 symptom clusters: intrusion, avoidance, negative alterations in cognitions and mood, and alterations in arousal and reactivity. This expansion recognizes broader, more heterogeneous symptom expressions (such as dysphoria and anger) while allowing for more dynamic changes in arousal and reactivity. Current treatments for PTSD are mainly psychotherapy based (e.g., exposure therapy and cognitive therapy). Pharmacological treatments, such as serotonin-selective and serotonin-norepinephrine reuptake inhibitors (SSRI/SNRIs), have also achieved modest efficacy (Committee on treatment of posttraumatic stress disorder IoMotNA 2007).

There is a clear need for the development of novel preventive and therapeutic treatment strategies for PTSD via increased understanding of etiological and maintaining factors of the disorder (Baker et al. 2009). To this end, there is a new focus on utilization of biological, physiological, and behavioral tools to enable a

"paradigm shift" from sole reliance on self-report measures to assess symptom status and diagnosis for psychiatric disorders such as PTSD. The US National Institute of Mental Health (NIMH) Research Domain Criteria project (RDoC) represents a framework for research in this area, with an emphasis on developing a diagnostic classification scheme based upon valid observable markers of common biological processes across the range of currently identified diagnostic categories. The negative valence system (NVS) domain suggested by the NIMH contains the constructs of acute threat of "fear," potential harm or "anxiety," and sustained threat. The 2011 NVS working group meeting identified many of the physiological measures reviewed below as important research tools for understanding these constructs. Psychophysiological measures may have utility as static markers of these constructs, as well as dynamic markers of change enabling the elucidation of the roles of learning and memory processes in the expression of these constructs. Thus, psychophysiological measures are poised to play an important role in the future understanding of mental illness generally, and traumatic stress-related disorder characterized by negative valence states more specifically.

Psychophysiological outcome measures have a number of advantages in neuropsychiatric research. (1) Psychophysiological measures provide objective, non-self-report outcomes and thus are less subject to bias by the subject and/or researcher. (2) Physiological measures are quantifiable. (3) Compared to self-report symptom scales, physiological measures may represent more discrete symptom domains that probe specific neurobiological pathways enabling mechanistic study of neurobiological abnormalities underlying symptoms. (4) Physiological measures enable cross-species translation to examine causal mechanisms of psychophysiological abnormalities linked to trauma exposure that cannot be achieved with self-report measures. The current manuscript will review the current state of knowledge on psychophysiological outcomes in PTSD with attention to their use as markers of current symptoms as well as markers of PTSD-related processes. We will also discuss these variables in terms of their sensitivity and selectivity for PTSD symptoms versus other anxiety and mood disorders and comorbid disorders such as traumatic brain injury (TBI). Further, we will discuss potential future avenues for integrating psychophysiology into emerging areas of PTSD research. We have limited our review to relatively common psychophysiological measures of arousal/threat, including cardiovascular, electromyographic, and electrodermal measures.

2 Psychophysiological Markers of Current PTSD Symptoms

2.1 Cardiovascular Activity

Baseline: Current conceptualizations of PTSD, reflected in the Diagnostic and Statistical Manual of the American Psychiatric Association (DSM 5; APA 2014) criteria, recognize that PTSD has a complex phenomenology expressed not just as

fear-based hyperarousal, but also as anhedonic and dysphoric emotional states. In contrast, earlier conceptualizations of the disorder, reflected in DSM-III through IV criteria, placed a larger emphasis on fear-related arousal. Given the past emphasis on arousal-related symptoms, research has long focused on identifying and understanding the psychophysiological basis of elevated arousal. Though studies have assessed the construct of arousal across a number of psychophysiological measures, an extensive body of work has focused on the cardiovascular system. Cardiovascular physiology is a convenient domain to focus on since it can be measured relatively easily using a number of different methods and equipment typically present in an emergency department or urgent care clinic. Further, some elements of cardiac physiology can be interpreted as a readout of sympathetic/parasympathetic balance, which has long been theorized to be disrupted in PTSD (see below).

Blanchard et al. (1982) observed that Vietnam veterans with PTSD had higher resting baseline heart rate (HR) and blood pressure (BP) than Vietnam veterans without PTSD. These initial observations were later largely confirmed in a meta-analysis by Buckley and Kaloupek (2001), which reviewed 34 studies of resting cardiovascular activity in PTSD conducted up to that time. This meta-analysis found support for elevated resting HR and diastolic blood pressure (BP), though systolic BP levels were similar across PTSD subjects and healthy controls. A more recent meta-analysis of psychophysiological studies in PTSD (Pole 2007) reviewed 55 studies conducted until that time and also supported increased resting HR in PTSD relative to healthy controls. However, elevations in systolic and diastolic BP were only present under relaxed criteria for statistical significance.

While the evidence for altered cardiovascular activity at rest in PTSD appears fairly strong, some researchers have suggested a more nuanced relationship. First, some studies (i.e., Shalev et al. 1992) have failed to find HR differences in new-onset PTSD. Further, Buckley and Kaloupek (2001) showed a greater effect size for HR in patients with chronic PTSD (>13 years). Taken together, these findings suggest that elevated HR may be a consequence of physiological changes driven by long-term PTSD. Second, studies monitoring cardiovascular activity over 24-h periods have suggested that HR and BP may fluctuate widely across the day, complicating previous studies (Muraoka et al. 1998; Buckley et al. 2004). One study using 24-h HR monitoring did, however, confirm increased HR in veterans with PTSD, with more pronounced effects during the night, perhaps related to the sleep disturbances commonly associated with PTSD (Agorastos et al. 2013). Third, there is disagreement among researchers regarding whether resting state activity is actually being measured in these studies, or if what is actually being captured are cardiovascular responses to a stressful situation/challenge induced by the testing environment (see below; Zoladz and Diamond 2013). Other studies suggest that PTSD subjects are hyperresponsive to stress or threat across a number of physiological markers, including HR, startle, and skin conductance (see below). Further, increased HR is not specific to PTSD, but is also reported in panic disorder and depression (Cohen et al. 2000; Blechert et al. 2007; Kamphuis et al. 2007).

An additional marker of resting-state cardiovascular activity that is altered in PTSD is heart rate variability (HRV). HRV is a measure of the variation in time between heart beats, which indicates autonomic flexibility (the higher the variation, the more flexibility). HRV is most accurately measured via electrocardiogram; however, photoplethysmography is also utilized. HRV is measured as time-domain variables (e.g., changes in the standard deviation of beat–beat interval) and frequency domains using power spectral density analysis methods. Frequency components are thought to represent sympathetic and parasympathetic control over HR, with the high frequency domain (HF; 0.15–0.4 Hz) representing parasympathetic or vagal tone, while the low frequency (LF; 0.04–0.15 Hz) is comprised of both parasympathetic and some sympathetic elements (see Heathers 2014; Berntson et al. 1997 for review). Finally, respiratory sinus arrhythmia, HRV due to respiration, is another measure of vagal control of autonomic activity. Reduced HRV is associated with mortality and cardiovascular symptoms in patients with PTSD, highlighting the clinical importance of these measures (Kubzansky et al. 2007). There is growing evidence that both LF and HF are reduced in PTSD patients, which may be suggestive of an imbalance between sympathetic and parasympathetic drive on cardiovascular output (Cohen et al. 2000; Blechert et al. 2007; Jovanovic et al. 2009), though exceptions have been reported (Sahar et al. 2001). In a recent twin study of combat-related PTSD in Vietnam era veterans, Shah et al. (2013) found that HRV abnormalities (lower LF and HF) were present only in the twin with PTSD, suggesting that reduced HRV is an acquired consequence of the disorder. They also suggested that HRV abnormalities were not present in subjects with remitted PTSD, suggesting HRV reductions are indicative of symptom state. We have recently shown that HRV reductions (reduced HF) are also associated with new-onset PTSD symptoms in active duty marines who served in Iraq/Afghanistan, suggesting that reduced HRV is not related to age or chronicity of PTSD (Minassian et al. 2014). These studies have also shown that reductions in HRV in these populations are not due to depression or TBI, nor are they related to degree of combat exposure or deployment history per se (Shah et al. 2013; Minassian et al. 2014). Finally, reduced HRV is reported in untreated subjects (Minassian et al. 2014; Chang et al. 2013), indicating that this phenotype is not due simply to medication side effects. Although HRV measures appear to be sensitive to PTSD symptoms, they are not specific to PTSD. Indeed, reduced HRV, in particular HF, may be a more general marker of anxiety disorders (Pittig et al. 2013) or even mental illness, as it is reduced across multiple disorders including anxiety, depression, bipolar disorder, and schizophrenia (Moon et al. 2013). It is possible that multiple mechanisms underlie the reductions in HRV across these diverse patient groups, or that reductions in HRV are due to the higher stress or allostatic load experienced by those with neuropsychiatric illness (McEwen 2000).

Response to Challenge: In contrast to resting-state cardiovascular markers, several studies have assessed cardiovascular activity in response to challenges from either loud acoustic stimuli (startle) or trauma-related cues. A large body of literature documents larger HR reactivity to startling sounds in PTSD patients (Pallmeyer et al. 1986; Shalev et al. 1992; Orr et al. 2002). Pole (2007) investigated 10 studies

measuring HR response to loud acoustic stimuli and found that elevated HR response was among the most robust effects found using this paradigm. Pitman et al. (2006) examined elevated HR reactivity to sudden loud tone presentation in a twin sample of Vietnam veterans. They found elevated HR reactivity only in the twin with PTSD, indicating HR response is an acquired consequence of the disorder rather than a predisposing trait.

HR response to trauma-related reminder cues has also been examined, which may probe biological mechanisms relevant to fear memory processes. These studies typically involve either "standardized" cues, such as combat sounds (Liberzon et al. 1999) that are held constant across the sample being studied, or "ideographic" cues which are tailored to be specific to each subject's traumatic experience. Pole (2007) reviewed 16 studies investigating HR response to standardized trauma cues and another 22 investigating HR response to ideographic trauma-related cues. Elevated HR response to standardized cues in PTSD emerged as one of the more robust effects in these paradigms. Support for increased HR responses to ideographic trauma cues was also found, though less robust than that for standardized cues. Recent studies have also supported these findings in both standardized (Adenauer et al. 2010; Suendermann et al. 2010; Ehlers et al. 2010) and ideographic trauma cues (Barkay et al. 2012). Barkay et al. (2012) have investigated the neurobiological correlates of this effect using PET imaging and found correlations between HR and rCBF in the orbitofrontal, precentral, and occipital regions of the cortex only in patients with PTSD and not in trauma-exposed non-PTSD subjects. These findings are suggestive that increased HR responses to trauma reminders may overlap in neural substrates (orbitofrontal cortex) with the reduced ability to inhibit fear responses (Shin et al. 2006). In PTSD, there are correlations between HR response to trauma and norepinephrine concentrations in cerebrospinal fluid (Geracioti et al. 2008), suggesting that central noradrenergic hypersignaling could play a role in this phenotype. It is unclear whether increased HR or other cardiovascular abnormalities are ameliorated by treatment, however, despite the use of noradrenergic reuptake inhibitors (Hoge et al. 2012) as well as clinical trials of the alpha 1 receptor antagonist prazosin (Raskind et al. 2013). Whether increases in HR are an epiphenomenon of increased centrally mediated fear responses, or are a core feature of PTSD pathology is unclear. One intriguing recent finding suggests that inhibitors of angiotensin I signaling, commonly given for hypertension, are associated with fewer PTSD symptoms in a cross-sectional sample of highly traumatized civilian populations (Khoury et al. 2012). Other common hypertension medications were not associated with fewer symptoms, suggesting that the angiotensin pathway may play a role in PTSD-related pathology. Thus, more research is clearly needed to further elucidate pathways involved in elevated cardiovascular responses in PTSD.

Summary of Cardiovascular Markers of PTSD Symptom State: Cardiovascular physiology is an active and important area of research in PTSD, especially given reported links between PTSD and increased incidence of cardiovascular disease (Wentworth et al. 2013). While there is strong evidence that resting-state cardiovascular activity, as well as HR response to standardized and ideographic trauma cues, is altered in PTSD, this is still an active area of research that is not without

controversy. Specifically, the degree to which the testing situation contributes to findings of elevated HR in PTSD is unclear. The extent to which elevated HR is a feature of core PTSD pathology versus simply a consequence of chronic stress is also unknown. Some studies have suggested that HR soon after trauma may predict development of PTSD, suggestive of HR being a proxy for biological risk factors for PTSD (see below). However, a recent study suggests that HR is not altered in relatively "recent" PTSD cases after combat (Minassian et al. 2014), arguing against elevated HR as a risk factor. HR increases are also not specific to PTSD, but are increased in other anxiety disorders more generally. Research investigating the time course and neurobiological correlates of altered cardiovascular activity in PTSD is needed to further clarify these issues.

Many questions still remain for the association of HRV with PTSD symptoms. Although twin studies suggest that altered HRV is specific to PTSD symptom state, prospective studies are needed to confirm HRV measures as symptom dependent or markers of risk for PTSD (Baker et al. 2012). Similarly, although there is some evidence from cross-sectional analysis in small samples for symptom remission to be associated with normalization of HRV (Shah et al. 2013), longitudinal treatment studies are required to best address this question. The biological mechanisms responsible for HRV reductions in PTSD are also unclear. However, dysregulated sympathetic output (e.g., via increased noradrenergic tone, Geracioti et al. 2001, 2008; Pietrzak et al. 2013) and abnormalities in stress and immune systems have been identified as candidate mediators (Risbrough and Stein 2006; Eraly et al. 2014).

2.2 *Exaggerated Startle Response*

Baseline: The startle response is a sensitive, noninvasive measure of central nervous system activity that is typically accessed via electromyographic (EMG) measurement of strength of contraction of the *orbicularis oculi* muscle controlling eyeblink in response to a sudden acoustic or tactile stimulus (Blumenthal et al. 2005). Exaggerated startle is a symptom of PTSD according to the DSM 5 (APA 2014). Thus, it follows that larger baseline startle responding should be detectable in PTSD. However, evidence for increased startle reactivity under "baseline" conditions in PTSD is mixed, with some studies finding evidence for increased startle in PTSD relative to healthy controls and others finding equivalent startle responses (see Zoladz and Diamond 2013 for a recent review of this literature). There are also some suggestions that increases in baseline startle may only occur in chronic PTSD patients or following certain forms of trauma, such as combat (Grillon and Baas 2003). A significant problem with assessments of "baseline" startle is that it is very difficult to accurately assess this phenomenon. Startle reactivity is extremely plastic, and it is sensitive to many rapid and dynamic modes of inhibition such as habituation and sensorimotor gating, to emotional valence or experimental context, and of course is extremely sensitive to stimulus parameters such as intensity and duration of the startling stimulus, all of which will influence the detection of putative differences.

For example, startle is higher in PTSD patients under low-intensity startle stimuli but not high intensity (Butler et al. 1990), which may reflect a lowering of startle thresholds rather than an exaggeration of startle responses elicited by supra-threshold stimuli. Thus, more robust and reliable startle phenotypes in PTSD and other disorders are measured when comparing startle across multiple stimulus conditions and emotional contexts. Startle has also generally only been explored in terms of magnitude of the response (muscle contraction) compared to controls. However, self-reports of "increased startle" from patients may not simply reflect magnitude, but *the probability* of a response under subthreshold conditions, which has yet to be explored.

In Response to Challenge: Given the inconsistency of baseline startle changes in PTSD, it has been suggested that startle reactivity is higher in PTSD patients only when under threat; thus, this phenomenon is indicative of mechanisms related to increased stress responding rather than disruption of baseline arousal (Grillon and Baas 2003). Grillon et al. (1998) reported normal baseline but increased startle magnitude in Vietnam combat veterans with PTSD during anticipation of experimental electrical shock relative to non-PTSD veterans, demonstrating a higher response in situations of threat or stress in PTSD. Startle is also elevated in response to trauma reminders (imagery, trauma scripts) in PTSD patients (e.g., Cuthbert et al. 2003; McTeague et al. 2010); however, these tasks are relatively unique to individual laboratories and more difficult to generalize across studies. As a whole, these studies suggest that exaggerated startle in PTSD is not indicative of increased arousal at baseline, but is a physiological marker of heightened response to threat and heightened fear responses in the presence of trauma cues. Thus, startle is increasingly used as a quantitative measure of fear responding that complements self-report data on anxiety and stress to identify biological mechanisms underlying PTSD symptoms.

Studies have recently suggested that elevated startle to challenge in PTSD may be subject to gender differences. Kamkwalala et al. (2012) showed that women with PTSD had higher startle in a dark environment relative to a light environment than men and women without PTSD. However, this elevated "dark-enhanced" startle was not present in male subjects with PTSD. Further, dark-enhanced startle has been shown to be associated with pituitary adenylate cyclase-activating polypeptide receptor (PAC1) genotypes in females, a gene that interacts with estrogen and has also been associated with PTSD in females (Ressler et al. 2011). These studies represent a new avenue of PTSD research that is just coming to fruition in utilizing physiological markers as intermediate phenotypes to identify biological pathways related to PSTD risk.

Startle Habituation: Habituation is a non-associative learning process whereby an organism displays a reduction in some innate orienting or defensive response following repeated presentation of a stimulus (Halberstadt and Geyer 2009). Shalev et al. (2000) examined habituation of the startle and electrodermal response to loud acoustic stimulus in a sample of traumatized Israeli civilians tested at 1 week and 1 and 4 months following the traumatic event. Those who developed PTSD began to show reduced habituation in both measures beginning 1 month post-trauma, suggesting that reduced habituation may be an acquired sign of PTSD. The reduced

startle habituation finding is confounded, however, as the methodology used to detect startle was flawed, with sample rates that were much too slow (50 Hz) to visualize the very fast on and off rate of a startle response which is typically measured with 1,000 Hz sampling rates. The reduced electrodermal habituation, however, supported earlier findings by this group (Shalev et al. 1992). Other studies had failed to detect reduced startle habituation in PTSD but were compromised by their use of inappropriately slow sampling rates (Pitman et al. 1987, 1993; Orr and Pitman 1993). A more recent study in Croatian combat veterans found that PTSD and control groups did not differ in startle habituation as assessed by quantitative analysis of EMG reduction across trial; however, there was a reduction in PTSD subjects compared to controls when using nonparametric comparisons of a number of subjects who met criteria for habituation (lowest responding at the last trial) (Jovanovic et al. 2009). This study also did not replicate habituation of the electrodermal response, a physiological marker of sympathetic nervous system arousal based on electrical conductivity across the skin due to sweat (see below). Thus, taken together across studies, evidence for differences in startle habituation in PTSD subjects is weak. PTSD subjects may exhibit reduced habituation of fear-potentiated startle during fear association training (Ressler et al. 2011). However, it is unclear whether this effect reflects reduced habituation to startling sounds or increased reactivity to the aversive stimuli used during fear conditioning. Reductions in habituation have been detected in other neuropsychiatric disorders (schizophrenia, panic disorder); thus, it is possible that reductions in habituation of the response may represent a pathology in a subset of patients across disorders, as such a phenotype would have substantial consequences for multiple behavioral functions (Geyer and Braff 1982; Ludewig et al. 2002a, b, 2003, 2005). Habituation is another "intermediate phenotype" that is being used to identify potential gene pathways disrupted in these disorders (Greenwood et al. 2012, 2013).

Prepulse Inhibition of the Startle Response: Prepulse inhibition (PPI), the unlearned suppression of the startle reflex to an intense acoustic stimulus when immediately preceded by a weaker acoustic prepulse, is an operational measure of sensorimotor gating (Geyer et al. 1990; Geyer and Braff 1987). PPI has been shown to be a robust but non-specific biomarker of psychiatric diagnosis. PPI performance is reduced compared to healthy controls in a number of neuropsychiatric disorders including panic disorder, obsessive compulsive disorder, schizophrenia, bipolar disorder, Tourette's disorder, and Huntington's disorder (Braff et al. 2001; Swerdlow et al. 2006; Castellanos et al. 1996; Perry et al. 2001; Ahmari et al. 2012; Ludewig et al. 2002a, b). Many of these disorders are linked to cortico-limbic circuit abnormalities (Kohl et al. 2013). Given the evidence for PTSD to have disruptions in this circuit (Shin et al. 2006), PPI in PTSD subjects has also been examined. However, PPI associations with PTSD are inconsistent, with some studies showing significantly reduced PPI in PTSD patients (Ornitz and Pynoos 1989; Grillon et al. 1996, 1998), while others detected no differences or only marginal differences (Butler et al. 1990; Morgan et al. 1997; Lipschitz et al. 2005; Holstein et al. 2010; Vrana et al. 2013). Thus, additional research is needed to clarify or refute the presence of PPI deficits in PTSD.

Summary of altered startle plasticity in PTSD: Exaggerated startle responding in PTSD patients is seen fairly consistently, most predominantly under conditions of challenge or threat. Pole (2007) conducted a meta-analysis of 20 studies measuring startle response via *orbicularis oculi* EMG both at baseline and after manipulation of contextual threat. This analysis supported a significant increase in startle responses in PTSD; however, this effect was not as robust as elevated cardiovascular responses. Furthermore, increased startle response to threat is also not specific to PTSD, but is also reported in other disorders that are characterized by high physiological arousal and fear (e.g., panic disorder) but not generalized anxiety disorder (Grillon et al. 2009; Grillon 2008). These findings suggest that disorders characterized by exaggerated startle may share an overlapping biological pathway. It is not clear, however, whether these effects are due to increased fear responses per se (e.g., via increased amygdala and/or insula circuit activation), or reduced ability to inhibit or modulate these responses appropriately (e.g., reduced modulation of amygdala output by hippocampal and cortical circuits; see below; Acheson et al. 2012; Klumpers et al. 2007).

Habituation and PPI are both measures of fundamental aspects of information processing that are disrupted in a number of psychiatric disorders and are to some degree heritable (Greenwood et al. 2007). However, there is relatively weak evidence at present for disruptions in PTSD. It is possible that disruption in these processes may indicate one of potentially many biological risk traits for neuropsychiatric disorders. Hence, further understanding of the genetic and neurobiological mechanisms underlying these phenotypes and their relationship to PTSD risk is worth further investigation. Indeed, PTSD is thought to share polygenic risk with other disorders that exhibit information processing deficits, such as bipolar disorder and schizophrenia (Nievergelt et al. in review; Solovieff et al. 2014).

While exaggerated startle per se is not unique to PTSD, it nonetheless represents a powerful method for exploring mechanisms underlying the development of PTSD symptoms. In animals, exaggerated startle phenotypes have long been utilized to test causal hypotheses of potential mechanisms underlying development of anxiety and fear-related behaviors after severe stress, including corticotropin-releasing factor and noradrenergic abnormalities (e.g., Risbrough and Stein 2006; Davis et al. 2010; Grillon et al. 2009). In humans, utilization of startle plasticity as an intermediate phenotype is just now beginning to be exploited (Greenwood et al. 2012). Further, questions of exaggerated startle magnitude versus reduced startle threshold in PTSD remain to be answered (Butler et al. 1990). Finally, surprisingly few pharmacological studies have thus far utilized startle to examine potential biological mechanisms of increased physiological responses in PTSD. Using a pharmacological challenge with the alpha 2 antagonist yohimbine, Morgan et al. (1995) showed that startle reactivity in PTSD patients may be via increased sensitivity to noradrenergic signaling.

2.3 Other Physiological Measures

Electrodermal Level/Response: In addition to HR and startle, researchers have examined electrodermal levels in PTSD both at resting baseline and in response to challenge. Electrodermal response, or the increase in electrical conductivity across the skin due to sweat, is a physiological marker of sympathetic nervous system arousal. A meta-analysis by Pole (2007) looked across 31 studies that measured resting electrodermal levels in subjects with PTSD versus controls and found support for significantly higher levels associated with PTSD, although the effect size was small. Blechert et al. (2007) found that PTSD subjects had higher resting baseline electrodermal level relative to both healthy controls and subjects with panic disorder, suggesting some diagnostic specificity. Resting electrodermal level has historically been reported to be reduced in subjects with depression versus healthy controls (Argyle 1991), further suggesting that this measure may hold some diagnostic specificity.

Electrodermal response to challenge by standardized and ideographic trauma cues has also been examined in relation to PTSD. Pole (2007) looked across 22 studies and found medium effect sizes for elevated electrodermal response to both standard and ideographic cues in PTSD versus controls. Interestingly, Blechert et al. (2007) found blunted electrodermal response in PTSD when subjects were under threat of electrical shock, suggesting that there may be a difference in effect between challenge by reminder cue versus challenge by contextual threat (experimental shock). Similarly, McTeague et al. (2010) found that PTSD subjects with multiple traumas and more severe, chronic PTSD showed blunted defensive responses to ideographic imagery. More recently, Glover et al. (2011) showed overall elevations in fear-potentiated startle in a classical conditioning paradigm in PTSD subjects relative to controls; however, no differences were found in electrodermal responses. It is possible that startle reactivity measures may offer a wider measurable range to detect increased reactivity than skin conductance measures because startle baseline can be controlled by the experimenter (i.e., via adjustments of the intensity of acoustic pulse). Thus, it is possible that startle may be more sensitive to detecting differences in responses even under relatively high arousal states (e.g., under threat). Skin conductance, however, offers other significant advantages over startle, since it does not require a relatively invasive stimulus (e.g., acoustic pulse) for measurement. The passive nature of this measurement has also supported its use as a complementary tool in imaging studies in which subject movement must be severely limited (i.e., startle response movement can disrupt image processing).

Facial EMG: Facial EMG has been used as a physiological measure of emotional response and typically involves measurement of activity in the frontalis, corrugator, and zygomaticus major muscles involved in emotional facial expressions such as smiling and frowning. Pole (2007) found support for increased frontalis and corrugator EMG activity while viewing ideographic trauma cues (12 and 5 studies, respectively). Pole (2007) found no support for altered facial EMG activity at resting baseline, or in response to standardized trauma cues (12 and 6 studies, respectively).

Because these measures are (1) more sensitive to artifact (e.g., non-specific facial and head movements, talking) and (2) are not easily controlled or evoked parametrically compared to reflexive responses such as changes in HR, skin conductance, and startle, they have not been utilized widely. They do not offer cross-species translation nor have well-defined circuits; thus, they may have less utility in understanding biological mechanisms of PTSD.

Summary of Other Physiological Measures Associated with PSTD: Elevated resting-state electrodermal level may be a psychophysiological measure that is relatively specific to PTSD. However, this measure is susceptible to the same methodological difficulties as resting HR or baseline startle response, namely that it is difficult to eliminate contextual factors that may influence stress and thus electrodermal activity. Electrodermal response to challenge presents a complicated picture with findings varying dependent upon both subject-specific and testing protocol variables. There is support for an association between increased facial EMG reactivity specifically in response to idiographic trauma cues; however, the utility of this measure for further biological research is limited.

3 Psychophysiological Markers of PTSD-Relevant Constructs: Fear and Sustained Anxiety

Safety Signal Learning: Safety signal learning is the process by which an individual learns to inhibit a learned fear response in the presence of a cue signaling absence of danger. This process is directly relevant to PTSD phenomenology insofar as PTSD is in part characterized by altered reactivity to trauma-related cues even in "safe" environments. Safety signal learning can be measured by assessing responses to a CS− that is never associated with an aversive event versus a CS+ that is contiguous with an aversive event, or via a specific CS that predicts absence of the aversive event when given in conjunction with the CS+. Using the latter paradigm, Jovanovic et al. (2010) recently tested this process in a sample of trauma-exposed civilians who were healthy, had PTSD, had major depression, or had comorbid PTSD and major depression with fear-potentiated startle as the primary outcome variable. Subjects learned that a cue predicted a blast of air to the throat, but that when this cue was presented along with another cue (the safety signal), the blast of air would not occur. Subjects with PTSD and comorbid PTSD/major depression failed to show inhibition of the potentiated startle response in the presence of the safety cue. Inability of subjects with PTSD to inhibit responding to a safety signal was also confirmed in the former paradigm, a simple CS+/CS− discrimination learning task (Jovanovic et al. 2013). Andero et al. (2013) found associations between the ability to learn to discriminate between the CS+ (danger) and CS− (safety) are impaired in subjects with a single nucleotide polymorphism (SNP) on the opioid receptor 1-like gene which encodes for the amygdala nociception/orphanin FQ receptor involved in pain processing. This SNP was also associated with greater PTSD symptoms, providing

further evidence for impaired safety signal processing in PTSD as well as a putative biological pathway for this effect. These results, though preliminary and in need of replication, suggest that failure to learn to distinguish between environmental cues signaling danger versus safety may be an important process that is impaired in PTSD.

Fear Extinction: Fear extinction is the process by which an organism learns that a cue that once signaled threat no longer does so, thus resulting in a progressive reduction in defensive physiological responding in the presence of this cue. Extinction of psychophysiological fear responding has long been considered a putative model of PTSD process due to its similarity to naturalistic recovery from trauma experience. Orr et al. (2000) and Peri et al. (2000) showed that PTSD patients failed to extinguish a conditioned electrodermal response to a cue signaling electrical shock or loud acoustic stimuli, respectively. Subsequent studies using electrodermal responses as the dependent variable have largely supported these original findings (e.g., Wessa and Flor 2007; Blechert et al. 2007). Norrholm et al. (2011) examined fear extinction in PTSD using fear-potentiated startle to a cue signaling an aversive air puff to the throat and found that PTSD patients showed greater potentiated startle in the early and middle portions of extinction training. This finding suggests that enhanced initial fear conditioning produced a greater "fear load" that the PTSD patients had to extinguish. This increased fear responding is also associated with specific symptom clusters of PTSD, re-experiencing (Glover et al. 2011), indicating this paradigm likely probes neural mechanisms of trauma memory.

Not all studies have found evidence for delay of fear extinction learning in PTSD. Milad et al. (2008) found equal levels of extinction performance, as measured by electrodermal response, in combat-related PTSD compared to combat-exposed monozygotic twins without PTSD and controls. However, the PTSD twins failed to recall this fear extinction learning when tested 24 h later. These results suggest that PTSD is not associated with a fear extinction learning deficit, but rather a fear extinction memory deficit. Further, this deficit appears to be an acquired sign of PTSD rather than an inherited trait. This difference in within-session learning results across these studies may be due to the physiological measures of fear used, startle versus skin conductance. The higher magnitude of the startle response to the conditioned cue in PTSD patients is providing a behavioral window to detect reduced/delayed extinction within session, which is not detectable via skin conductance responses (Glover et al. 2011). Taken together, these data suggest overall that there is higher fear responding in PTSD patients, which subsequently takes longer to extinguish fully and is less likely to be fully extinguished upon retesting. Additional research will be needed to determine the time point at which extinction deficits may occur, the most effective method for capturing such deficits, and the specific role these deficits play in PTSD symptomatology.

Summary of Psychophysiological Markers of PTSD-relevant Constructs: Psychophysiological markers have emerged as critical measures of unbiased fear responding to understand fear and anxiety domains disrupted in PTSD. These markers provide quantifiable assessments of autonomic processes that may not be adequately probed by self-report. They have been critical behavioral measures that

complement studies of the neural circuits underlying PTSD pathology, such as cortico-hippocampal–amygdala circuit function (Quirk et al. 2006), that can be translated across species for further study of causal factors for PTSD symptoms or PTSD risk. The intriguing preliminary evidence for safety signal learning to be disrupted specifically in PTSD versus depression patients may indicate this is a potential "biomarker" of PTSD, but needs further research and replication. Extinction has shown to be impaired in a number of neuropsychiatric disorders as well as PTSD, including obsessive–compulsive disorder and schizophrenia (Holt et al. 2009; Milad et al. 2013), suggesting that extinction learning may probe common pathological circuits across these disorders. Impairment in these processes is further supported by imaging research showing impaired function and structure of the ventromedial prefrontal/orbitofrontal cortex in PTSD subjects, which are structures known to be central to fear extinction learning and memory (Shin et al. 2006). Recent research suggests involvement of these areas in safety signal learning as well (Jovanovic et al. 2013). Finally, more recently, these paradigms have been utilized in healthy controls or PTSD patients to serve as proof of concept tests for novel treatments for fear-related disorders such as PTSD, with recent or ongoing tests of cannabinoid agonists (Rabinak et al. 2013), oxytocin (Acheson et al. 2013), glucocorticoids (de Quervain et al. 2011), and dopamine agonists (Haaker et al. 2013), among others. It remains to be determined how predictive these paradigms will be for treatment efficacy; however, this is an exciting avenue for PTSD drug discovery.

4 Psychophysiological Markers of Risk for Developing PTSD Following Trauma

Trait Markers: Given that elevated physiological reactivity is a common finding in those with current PTSD, researchers have explored the possibility that this elevated reactivity might serve as a marker of risk prior to or immediately following the traumatic experience. Several studies examined the relationship between HR shortly following trauma and later development of PTSD and found that elevated HR following trauma predicted development of PTSD symptoms (Bryant et al. 2000; Kassam-Adams et al. 2005; Shalev et al. 1998; Zatzick et al. 2005; Kuhn et al. 2006; Gould et al. 2011). Though numerous exceptions have been reported (Blanchard et al. 2002; Buckley et al. 2004; Ehring et al. 2008; Roitman et al. 2013; Price et al. 2014). In a related study, Suendermann et al. (2010) found that HR response to trauma-related images in motor vehicle accident survivors 1 month after trauma predicted PTSD severity at 6 months after trauma. The inconsistency in these findings may be due to the fact that cardiovascular activity assessed immediately post-trauma in the ambulance or emergency department may be subject to too many contextual variables, methodological inconsistencies, or ceiling effects that may limit reproducibility of findings. Newer technology allowing for ambulatory monitoring in the days following trauma (see below) may prove more useful

in determining at which time points and under what circumstances post-trauma HR may be most predictive of future PTSD.

While these studies of peri-traumatic HR suggest potential clinical utility as a marker of risk in traumatized individuals, they tell us little about who might be at risk for trauma before the event happens. Toward answering this question, Pitman et al. (2006) examined HR responses to a series of loud tones in Vietnam veterans with PTSD and their non-combat-exposed monozygotic twins. Only the twin with PTSD showed elevated HR response relative to combat-exposed veterans without PTSD and their non-exposed twins, suggesting that elevated HR response is an acquired sign of PTSD rather than a risk factor. However, further longitudinal studies where HR response is measured prior to trauma will be necessary to definitively rule out HR as a prospective marker of risk for PTSD. Pole et al. (2009) measured a number of physiological indices (startle, electrodermal response, HR) in response to startling tones under conditions of varying contextual threat (low, medium, and high threat of electrical shock) in new police academy cadets. These cadets were then later assessed for PTSD symptoms following one year of police work. They found that elevated startle measured by eyeblink EMG (with appropriate sampling rate), elevated electrodermal response, and slower habituation of the electrodermal response predicted PTSD symptom severity, but that HR response did not. Further, the associations between physiological reactivity and PTSD severity varied as a condition of the contextual threat: Greater electrodermal response was associated with PTSD symptom severity under low and high threat, and eyeblink EMG under medium threat was associated with symptom severity. These findings support the hypothesis that increased physiological reactivity to threat may be a useful marker for understanding biological mechanisms of PTSD risk.

Markers of Fear and Anxiety Constructs: Little is known about how abnormalities in safety learning and fear extinction may function as preexisting markers of risk for PTSD. A recent study found that impaired ability to inhibit fear-potentiated startle responding in the presence of a safety cue was associated with PTSD symptoms 2 and 9 months after combat-related trauma (Sijbrandij et al. 2013). These findings suggest that impaired safety signal learning may be important in predicting the maintenance of PTSD symptoms over time. It is not clear, however, whether reductions in safety signal learning predict PTSD prospectively. Investigators have also begun to look at impaired fear extinction processes as risk factors for developing PTSD following trauma. A twin study of combat-related PTSD by Milad et al. (2008) suggested that reduced recall of fear extinction memory is an acquired sign of PTSD rather than a preexisting risk factor. Guthrie and Bryant (2006) examined initial fear extinction learning of an aversively conditioned corrugator EMG response in a sample of firefighter trainees. They found that slower extinction while in training predicted PTSD severity after later exposure to trauma. Lommen et al. (2013) showed similar effects in a sample of Dutch combat veterans, though they only assessed explicit contingency awareness rather than physiological response. Further prospective–longitudinal studies assessing both habituation and extinction prior to trauma are needed to confirm whether or not these are robust markers of PTSD risk.

Summary of Risk Markers: While peri-traumatic physiological response may provide some information regarding who is at risk for developing chronic PTSD, more research is needed to solidify the extant findings and to link elevated physiology following trauma to specific biological changes underlying chronic disorder. Much less is known about using physiological markers to predict risk for PTSD prior to traumatic experience, though the results of Pole et al. (2009) provide promising avenues for future research in this area and suggest the possibility of achieving superior prediction by the integration of multiple psychophysiological domains into a single marker for risk. Knowing who is at risk for PTSD prior to trauma may have utility for screening of soldiers and first responders such as firefighters and police officers. Identification of pretrauma risk factors that are modifiable can inform prevention efforts in these and other populations at high risk for trauma exposure and may also point toward fruitful targets for novel treatment efforts.

5 Future Areas of Application for Psychophysiological Research

Psychophysiological Markers of Treatment Response: Beyond serving as markers of PTSD state or risk for developing the disorder, psychophysiological outcomes may have potential to provide objective markers of treatment response. This utility is particularly relevant as the NIMH now requires treatment studies to include biological and/or physiological markers along with standard symptom scales. To date, however, relatively few studies have made use of physiological outcome measures. To our knowledge, there are no reports of psychophysiological responses in PTSD patients during standard pharmacotherapies, e.g., serotonin reuptake inhibitors. Two recent studies using psychotherapy have included physiological markers. Robinson-Andrew et al. (2014) assessed potentiated startle responding in the presence of trauma-related visual cues in a small number of combat veterans with PTSD before, during, and after either prolonged exposure or "present-centered therapy" treatment. Treatment responders showed a dynamic pattern of increasing and then decreasing startle potentiation across treatment, while non-responders did not change. In another recent study, Rothbaum et al. (2014) compared the effects of d-cycloserine, alprazolam, and placebo on response to 5 sessions of prolonged exposure therapy for PTSD. Outcomes consisted of both self-reported diagnostic assessments as well as potentiated startle response to trauma-related images. The patients receiving d-cycloserine showed significantly lower startle potentiation post-treatment, and magnitude of startle reduction was associated with self-reported treatment response in this group only. However, groups did not differ on self-reported response to the treatment overall. There is no research yet on treatment effects on PTSD-related constructs of fear extinction or safety signal learning.

One earlier area of study where psychophysiological outcomes appeared promising was in predicting potential prophylactic efficacy of propranolol, a beta-adrenergic receptor antagonist. Pitman et al. (2002) originally showed that propranolol given immediately after trauma reduced physiological arousal (HR, electrodermal response, facial EMG) to script-driven traumatic imagery 3 months later, as well as showing a nonsignificant trend toward reduced PTSD symptom severity 1 month following trauma. In a larger study, Hoge et al. (2012) showed mixed results when propranolol or placebo was given to emergency department patients for 19 days following trauma. In "high-medication adherence" subjects, those who took the active drug showed reduced physiological reactivity to trauma imagery across three domains (electrodermal response, HR, lateral frontalis EMG) at 1 month following trauma relative to those who received placebo. However, this difference was not found at 3 months post-trauma, nor was there an effect of treatment on PTSD symptoms. Given the very mixed literature for treatment efficacy of propranolol as a prophylactic treatment for PTSD (Vaiva et al. 2003; Stein et al. 2007; McGhee et al. 2009), the predictive validity of psychophysiological measures for propranolol prevention of PTSD symptoms is inconclusive. Current studies have now shifted to examination of propranolol effects on memory reconsolidation in PTSD patients (www.clinicaltrials.gov), based in part on recent findings that propranolol given immediately after reactivation of the trauma memory via script preparation reduces physiological responding to the same script one week later (Brunet et al. 2009).

Psychophysiological outcomes have also seen limited use in studies investigating potential novel treatments. Jovanovic et al. (2011) showed that dexamethasone treatment reduces fear-potentiated startle in PTSD patients, suggesting that this treatment could reduce physiological symptoms of fear in these patients. These results provide preliminary support for the predictive validity of fear-potentiated startle in PTSD, since glucocorticoid agonists may reduce PTSD symptoms (Aerni et al. 2004; Steckler and Risbrough 2012). An ongoing study is also assessing the efficacy of corticotropin-releasing factor receptor antagonist treatment on both PTSD symptoms and fear-potentiated startle (Dunlop et al. 2014). We expect that more studies will utilize this complementary approach of physiological and self-report measures to assess treatment efficacy in the future.

Overall, psychophysiological outcomes have not been utilized in treatment studies and thus remain largely untested for sensitivity to treatment effects for PTSD. An important caveat is that some studies have shown a pattern of treatment-induced reductions in psychophysiological arousal, but not in self-reported PTSD symptom severity. This pattern of findings suggests several possibilities. First, psychophysiological alterations may not be powerful enough to generalize into symptom change per se (e.g., Hoge et al. 2012). Second, psychophysiological alterations may be one of the several potential mechanisms of change occurring within the same treatment protocol (e.g., Rothbaum et al. 2014). These conclusions suggest that psychophysiological assessment may be used as an objective marker of treatment response and have utility in elucidating mechanism/process of change that may vary across subjects being treated with the same protocol. Further, psychophysiological

assessment may have utility for understanding which patients may benefit from among several treatment modalities aimed at the same overt condition (Aikens et al. 2011). More research is required before this approach can be considered a realistic possibility in the near term.

Consideration of Mild Traumatic Brain Injury (mTBI) in Psychophysiological Investigations of Trauma-related Pathology: Many of the traumatic experiences that might result in development of PTSD (motor vehicle accident, physical assault, combat) also involve potential for physical harm. The large numbers of blast-related injuries coming out of the wars in Iraq and Afghanistan (Hoge et al. 2008) have brought into recent focus the potential relationship between mTBI and PTSD. A prospective study of service members deployed in these conflicts suggests a strong association between deployment-related mTBI and post-deployment PTSD symptoms (Yurgil et al. 2014). These findings suggest that mTBI may need to be considered as an important factor in assessing psychophysiological outcomes in PTSD, similar to its potential effects on neurocognitive symptoms in PTSD (Vasterling et al. 2009, 2012). Little research has been conducted on how mTBI affects the physiological markers discussed here, with the exception of HRV. HRV is reduced in some TBI patients, with alterations related to time since injury and injury severity (Keren et al. 2005; Baguley et al. 2006). One study in active duty marines with PTSD suggests that HRV is reduced in PTSD subjects even when controlling for TBI although TBI was also independently associated with reduced HRV (Minassian et al. 2014). Williamson et al. (2013) have suggested that in cases of mTBI-induced damage to white matter tracts involved in emotional behavior (e.g., uncinate fasciculus and the anterior limb of the internal capsule) may cause disruption of top-down control of autonomic nervous system activity reflected in psychophysiological measurements. These forms of disruption could also explain the higher risk for development of PTSD in individuals exhibiting mTBI (Yurgil et al. 2014). Interestingly, recent animal studies have also supported that mild TBI could result in sensitization of fear learning processes (Heldt et al. 2014). Thus, mTBI should be carefully considered in future assessments of PTSD-related physiology, particularly in abnormalities of cortical-mediated inhibitory processes and fear learning constructs, to understand its modulating or mediating role in psychophysiological abnormalities in PTSD.

Wearable Physiological Monitoring Technology: Although the specific physiological abnormalities linked to trauma symptoms are becoming more clear as reviewed above, one of the next steps for the field is to determine whether these measures can translate to clinical applications, such as prediction of symptom development, symptom class, and/or treatment response. Moving these measures to clinical applications faces significant hurdles, one of which is the development of more usable devices that are not dependent on narrow laboratory-specific parameters or expensive and complicated hardware. One potential area for psychophysiology variables in mental health in the future is use of "wearable" devices in subjects that have experienced, or at risk for, trauma (Darwish and Hassanien 2011).

There is a strong push both in private and academic medical sectors to implement wearable devices for a host of medical purposes including diabetes, cardiovascular disease management, cognitive therapy aids, and other lifestyle aids for better wellness. Predictive psychophysiological variables relevant to PTSD phenotypes that may be conducive to wearable technology are measures of physical activity via accelerometers (e.g., Fukukawa et al. 2004), sleep (Suzuki et al. 2014), skin conductance (Rajan et al. 2012), HR and HRV (Billeci et al. 2014), EMG (Grenier et al. 2012), and EEG (Zao et al. 2014). The development of these wearables will enable assessment of dynamics of physiology in naturalist settings, at rest (i.e., sleep) as well as during stress. These devices may help answer the question of which physiological variable, or combination of variables, might be able to predict development of PTSD symptoms after trauma exposure (e.g., after discharge from the ER/hospital). Another question is if physiological markers are sensitive to treatment, and when in the recovery process does this happen (i.e., could these markers serve as early predictors of treatment response?). Many of these variables are not "static," for example, longer-term assessment of sleep variance across multiple nights will enable a much more comprehensive picture than can feasibly be obtained in laboratory settings. Similarly, HRV over long time periods will provide greater fidelity in the assessment of cardiovascular changes after trauma. Some wearable devices may also be utilized in "at-risk" populations, such as rescue service and military personnel, to develop algorithms of risk based on physiological response and recovery after trauma exposure. This approach is currently being examined in the military (Tharion et al. 2013). However, a number of hurdles must be considered in terms of feasibility/practicality of the technology, the data quality, storage capacity, and of course the ethical component of resulting data being used or stored improperly.

One example of current status of technology is assessment of continuous HR. HRV can now be obtained via sophisticated wearable devices (e.g., pulse oximeter introduced into a wrist watch) over long periods of time with little burden to the subject. However, technical challenges must be addressed, including the high sampling rate needed for HRV assessments that can produce power and data storage limitations for continuous monitoring. Data quality is also affected significantly by movement artifact for many of these devices. Thus, despite significant promise, many technical limitations must be addressed before these devices will produce reliable physiological assessments for utility in prediction and intervention.

6 Conclusion

As discussed above, there are now a number of well-validated physiological phenotypes that are reliable across multiple studies/laboratories, including increased and poorly inhibited physiological responses to threat (electrodermal and EMG), as well as altered HRV. We are just now beginning to understand these measures in a larger context of symptom domains, as well as comorbid symptoms (depression, TBI, etc.).

Much more work is needed, however, to refine these phenotypes in terms of specific associations with PTSD symptoms versus other anxiety disorders and comorbid symptoms (depression, TBI). Importantly, many of these phenotypes are now well mapped to circuitry that supports translational research across species for mechanisms driving these phenotypes, which will support development of novel treatment targets. To this end, psychophysiological measures are increasingly being used as complementary measures for integration with both self-report and other biological assessments (e.g., blood-based or genetic markers). We expect much more research in the years to come with these tools for objective assessment of treatment outcome. Finally, in the long term, wearable technology could accelerate the feasibility of these markers as tools to identify risk and symptom development in clinical settings.

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Blood-based gene-expression biomarkers of post-traumatic stress disorder among deployed marines: A pilot study

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Summary: The etiology of post-traumatic stress disorder (PTSD) likely involves the interaction of numerous genes and environmental factors. Similarly, gene-expression levels in peripheral blood are influenced by both genes and environment, and expression levels of many genes show good correspondence between peripheral blood and brain tissues. In that context, this pilot study sought to test the following hypotheses: (1) post-trauma expression levels of a gene subset in peripheral blood would differ between Marines with and without PTSD; (2) a diagnostic biomarker panel of PTSD among high-risk individuals could be developed based on gene-expression in readily assessable peripheral blood cells; and (3) a diagnostic panel based on expression of individual exons would surpass the accuracy of a model based on expression of full-length gene transcripts. Gene-expression levels in peripheral blood samples from 50 U.S. Marines (25 PTSD cases and 25 non-PTSD comparison subjects) were determined by microarray following their return from deployment to war-zones in Iraq or Afghanistan. The original sample was carved into training and test subsets for construction of support vector machine classifiers. The panel of peripheral blood biomarkers achieved 80% prediction accuracy in the test subset based on the expression of just two full-length transcripts (*GSTM1* and *GSTM2*). A biomarker panel based on 20 exons attained an improved 90% accuracy in the test subset. Though further refinement and replication of these biomarker profiles are required, these preliminary results provide proof-of-principle for the diagnostic utility of blood-based mRNA-expression in PTSD among trauma-exposed individuals.

1. Introduction

Post-traumatic stress disorder (PTSD) is a severe anxiety syndrome that is currently diagnosed based on the emergence and persistence of core clinical symptoms including hyperarousal, re-experiencing, avoidance, or emotional numbing for a period greater than one month. Early psychosocial intervention after stress exposure may help reduce some of the symptoms and prevent the development of chronic PTSD (Litz et al., 2002). However, many individuals initially presenting with PTSD-like symptoms recover spontaneously and do not develop chronic PTSD (McFarlane, 1997). Thus, identifying which individuals will benefit most from early intervention can be challenging. Despite possible benefits of early intervention and a growing knowledge of the pathophysiology accompanying PTSD, a readily assessable diagnostic biomarker for PTSD has yet to be validated.

Classical descriptions of PTSD pathophysiology have included dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, but a specific pattern of dysregulation is not consistently observed across studies. Similarly, heightened inflammatory signaling has been reported in some (but not all) contexts (Baker et al., 2012b). Some have proposed a model of insufficient regulation of inflammatory signaling (Heinzelmann and Gill, 2013). Yet, there is an apparent paradox; *i.e.*, the observation that peripheral blood mononuclear cells (PBMCs) from PTSD patients show increased sensitivity to glucocorticoid-mediated suppression of an *in-situ* inflammatory response (van Zuiden et al., 2012b).

There is considerable evidence that genetic effects, environmental influences, and their interaction play a role in the development of PTSD (Afifi et al., 2010; Koenen et al., 2009; True et al., 1993). There is a well-established body of clinical literature supporting a link between early life events, previous exposure to traumatic stress, and other psychosocial factors with the development of PTSD (Brewin et al., 2000; Ozer et al., 2003; DiGangi et al., 2013). Many biological investigations of PTSD have focused on the HPA axis and glucocorticoid receptor (GR) signaling pathways. In a cross-sectional study, Binder et al. (2008) identified an interaction between child abuse history and genetic polymorphisms in *FKBP5* (a negative regulator of GR sensitivity) in predicting adult PTSD symptomology among a sample of non-psychiatric medical clinic patients. Mehta et al. (2011) described the association between genetic polymorphisms in *FKBP5* and dysregulated neuroendocrine profiles described in PTSD. van Zuiden et al. (2012a) provided evidence that increased GR density is a pre-trauma risk factor for the development of PTSD and that dysregulation of GR density may be associated with an interaction between polymorphisms in the GR gene and concomitant early life stress. Another line of research suggests that genetic variants in corticotropin-releasing hormone type 1 receptor (*CRHR1*) are a risk factor for PTSD in children who were abused at an early age (Gillespie et al., 2009). PTSD is thus thought to be a disorder whose development is influenced by multiple genetic and environmental effects that establish a susceptible biological state; this vulnerability may be reflected in gene-expression signatures.

In light of the less-than-absolute heritability of PTSD and the prominent role of environmental factors, the pursuit of static genetic markers alone (*e.g.*, single nucleotide polymorphisms and copy-number variations) likely will not yield a suitable diagnostic biomarker. Gene-expression (*i.e.*, mRNA) levels, which potentially reflect the effects of both heredity and environment, may be better indicators of the aberrant biology underlying PTSD. PTSD clearly is a brain disorder, but assaying gene-expression levels — either acutely or longitudinally — in the brains of living human subjects at risk for PTSD is impossible. Yet, peripheral blood expression levels of many genes are moderately correlated with

the expression levels of those genes in other tissues, including *postmortem* brain (Tylee et al., 2013) suggesting the possibility that peripheral blood gene-expression can be harnessed to construct useful profiles of brain disorders. Previous work by our group and by others has demonstrated that peripheral blood gene-expression provides a useful biomarker signal for a number of neuropsychiatric disorders, including schizophrenia, bipolar disorder, and autism spectrum disorders (Glatt et al., 2009, 2005; Tsuang et al., 2005).

In the context of PTSD, several prior studies identified differences in peripheral blood gene-expression levels between individuals with PTSD and similarly exposed comparison subjects without PTSD (Neylan et al., 2011; Segman et al., 2005; Yehuda et al., 2009; Zieker et al., 2007) (see Glatt et al., 2013 for a brief review of these studies). Taken together, these studies suggest that PTSD is associated with alterations in peripheral blood gene transcripts thought to play a role in HPA axis function, glucocorticoid signaling, immune and inflammatory signaling, and metabolism of reactive oxygen species. Consolidating this evidence with the results from a large body of epidemiologic, genomic, and neurobiological studies of the disorder (*e.g.*, Uddin et al., 2010) led us to recently propose a theory of PTSD predicated on dysregulation of immune and inflammatory processes in general, and cellular immunity in particular (Baker et al., 2012b). We maintain that a variety of specific genetic factors and environmental influences may play a role in producing this dysregulated immune and inflammatory phenotype within different individuals. For this reason, we propose that a blood-based diagnostic biomarker calibrated to detect commonly-dysregulated immune and inflammatory transcripts may ultimately provide the best sensitivity for detecting PTSD within a clinical sample. Our previous work in this domain supports this hypothesis and further proposes that pre-existing dysregulation of immune and inflammatory processes may dispose individuals to develop PTSD at some future time, following exposure to traumatic stress (Glatt et al., 2013). Another recent publication, examining a large group of Marine Resiliency Study subjects across multiple cohorts, provided strong evidence that pre-deployment inflammation levels, assessed *via* measurement of plasma C-reactive protein level, were a strong positive predictor for the development of post-deployment PTSD after controlling for other risk factors (Eraly et al., 2014).

In the context of this prior work, we report here the results of a pilot study examining transcriptome-wide expression-profiling of pre- and post-exposure peripheral blood samples from individuals with uniquely elevated rates of trauma exposure and PTSD development: participants in the Marine Resiliency Study (MRS) following return from active war zones in Iraq or Afghanistan, as part of an ongoing longitudinal investigation (Baker et al., 2012a). The objectives of this pilot study were to evaluate the following hypotheses: (1) post-trauma expression levels of some genes in peripheral blood cells would differ between Marines with PTSD and matched comparison subjects; (2) a readily assessable, predictive biomarker panel of the PTSD diagnostic status could be developed based on gene-expression levels in peripheral blood cells; and (3) a diagnostic panel based on the expression of individual exons would surpass the accuracy of a model based on the expression of full-length transcripts of genes. We interpret the results of these analyses in two contexts: (1) as a means of identifying biological functions, processes, pathways, and protein domains whose genomic dysregulation may indicate or influence the development of the disorder; and (2) as an approach to the construction of classifiers that might ultimately assist in the clinical diagnosis of PTSD in such populations.

2. Methods

2.1. Ascertainment and clinical characterization of subjects

The MRS is a prospective study of factors predictive of PTSD among approximately 2,600 Marines in four battalions deployed to Iraq or Afghanistan. The research team conducted structured clinical interviews on Marine bases and collected blood samples and data at four time points: pre-deployment, and 1-week, 3-months, and 6-months post-deployment. Measures collected, including those used in this study, have been described in detail previously (Baker et al., 2012a).

The principal exclusion criteria were identical to those used for the pre-deployment gene-expression studies (Glatt et al., 2013). Subjects were excluded if they showed clinically significant PTSD prior to deployment, manifesting in: 1) a pre-deployment PTSD Checklist (PCL) score >44; and/or 2) a pre-deployment diagnosis of PTSD based on the Clinician-Administered PTSD Scale (CAPS). PTSD cases were identified as those subjects who were issued a CAPS-based PTSD diagnosis at 3- and/or 6-months post-deployment. Unaffected comparison subjects were identified as those subjects who, at no time, attained a PCL score >44 and who were not issued a CAPS-based PTSD diagnosis at any post-deployment interview. Among subjects who were included in the full MRS sample and assigned to case or comparison groups based on these criteria, we then selected for analysis 25 male PTSD cases and 25 male comparison subjects based on similar demographics, pre-deployment clinical characteristics, deployment history, and levels of trauma exposure as determined from the combat and post-battle experiences subscales of the deployment risk and resilience inventory (DRRI). The group of subjects selected for this study largely overlapped with those featured in our previous study of pre-deployment gene-expression (Glatt et al., 2013); 24 of the 25 PTSD cases and 23 of the 25 comparison subjects within the present study were also featured in the pre-deployment study. The demographic, clinical, and combat-experiential characteristics of the subjects are shown in Table 1. The two groups were comparable on all demographic and combat-experiential variables. Within both the case and comparison groups, 50% of the subjects had previously been deployed on at least one occasion and the average number of previous deployments was not significantly different between the two groups. Although no subject met diagnostic threshold for PTSD at pre-deployment as determined by either clinician ratings on the CAPS or self-ratings on the PCL, the eventual PTSD cases did have significantly higher clinician ratings on the

Table 1 Demographic, clinical, and experiential characteristics of PTSD cases and non-PTSD comparison subjects.

	PTSD cases	Comparison subjects	p
Sample size: n	25	25	
Age:	22.4 ± 3.1	21.9 ± 3.1	0.576
Previously deployed: n (%)	13 (52.0)	13 (52.0)	1.000
Ancestry: caucasian n (%)	17 (68.0)	19 (76.0)	0.754
Cohort n (%): 1	3 (12.0)	5 (20.0)	0.721
2	8 (32.2)	8 (32.2)	
3	14 (56.0)	12 (48.0)	
DRRI combat experiences	18.5 ± 13.0	19.3 ± 14.8	0.846
DRRI post-battle experiences	7.25 ± 4.5	8.0 ± 4.5	0.518
CAPS pre-deployment	22.4 ± 118	14.0 ± 8.7	0.006*
CAPS 3-months post-deployment	62.8 ± 19.0	11.8 ± 10.8	<0.001*
PCL pre-deployment	24.3 ± 6.5	22.8 ± 3.4	0.330
PCL 1-week post-deployment	42.9 ± 17.2	23.0 ± 5.2	<0.001*
PCL 3-months post-deployment	49.0 ± 12.4	21.6 ± 6.1	<0.001*
PCL 6-months post-deployment	39.3 ± 15.0	19.8 ± 2.4	<0.001*

Notes: (1) demographic characteristics of each sample are reported as mean ± s.d. unless otherwise noted. (2) Sample means and proportions were compared using independent samples t-tests and chi-square tests, respectively. (3) p-Values < .05 are indicated with*.

CAPS at pre-deployment, whereas no significant difference in pre-deployment self-ratings on the PCL were observed

2.2. mRNA sample acquisition, stabilization, isolation, and storage

Close collaboration with the Marine Corps and the Navy, which provides health support for the Marine Corps, enabled comprehensive on-site data collection. Clinical interviews and sample blood draw (10 ml) were both performed within 4 h of each other on the same day, 3 months after return from deployment. Specific methods for stabilization, isolation and storage of mRNA samples were described previously (Glatt et al., 2013).

2.3. mRNA quantitation, quality control, and hybridization

Specific methods employed for mRNA sample quantitation and quality control assessment were also described previously (Glatt et al., 2013). The quantity and purity of mRNA in each of the 50 samples were sufficient for microarray hybridization assay. Two batches of 25 samples each (balanced with PTSD cases and controls) were then assayed on GeneChip® Human Exon 1.0 ST Arrays (Affymetrix, Inc.; Santa Clara, CA, USA) per the ''Whole Transcript Sense Target Labeling Assay'' protocol (Affymetrix, 2006) using 1 μg of total RNA from each sample.

2.4. Microarray data import, normalization, transformation, summarization, and quality control

Partek® Genomics Suite software, version 6.6© 2012 (Partek Incorporated; St. Louis, MO), was utilized for all analytic procedures performed on microarray scan data. Interrogating probes were imported, and corrections for background signal were applied using the robust multi-array average (RMA) method (Irizarry et al., 2003), with additional corrections applied for the GC-content of probes. The set of GeneChips was standardized using quantile normalization and expression levels of each probe underwent log-2 transformation to yield distributions of data that more closely approximated normality. As most genes were measured by multiple probe sets (typically one probe set per exon, but sometimes more), summarization of probes took place at two levels: first, probes tagging the same exon were summarized by median polish to arrive at one expression value per exon; second, exons tagging the same gene were summarized by median polish to arrive at one expression value per gene. All probesets were expressed with a signal:noise ratio ≥3; thus, no probesets were excluded from analyses of differential expression. A total of 257,106 probesets were analyzed, mapping to 28,536 whole transcripts and 253,002 exons. Unsupervised clustering of subjects revealed no evidence of batch effects based on scan date. Principal Components Analysis (PCA) of the 50 post-deployment data points identified no outliers; all 50 subjects' data were well within the four-SD ellipsoid on each of the first three PCA dimensions, and deviation among redundant probes located within the same chip was low.

2.5. Microarray data analyses

Four independent sets of analyses were performed on the microarray data, as described below. For analyses of covariance (ANVOCAs), nominal uncorrected p-value thresholds were selected in order to generate reasonably sized lists of differentially expressed genes and exons for biological annotation analysis and machine learning classifier construction.

2.5.1. Identification of differentially expressed genes and their associated biological terms

We utilized ANVOCAs to determine which full-length genetic transcripts were differentially expressed at post-deployment in peripheral blood cells between PTSD cases

($n=25$) and comparison subjects ($n=25$). We performed 28,536 ANCOVAs to assess each gene's expression level as a function of PTSD status (case or control), deployment cohort (three levels corresponding to three battalions deployed at different times), age, ancestry (dichotomized as Caucasian or not, as most subjects were Caucasian), and prior deployment status (first or subsequent deployment).

Family-wise Bonferroni-correction was applied to the ANCOVA p-values to determine whether any genes reached a genome-wide level of significance. To generate a relatively large candidate-gene list for functional profiling and construction of classifiers, we utilized an uncorrected type-I-error rate for diagnosis in these analyses at 0.01. We then reduced the dimensionality of the resulting list of candidate biomarkers through analysis of annotation-enrichment using the DAVID algorithm (Dennis et al., 2003) to determine if the gene list disproportionately represented any biological "terms". Details of the enrichment analysis are described previously (Glatt et al., 2013). Bonferroni-correction was applied to the p-values obtained in the enrichment analyses of these annotation terms.

Pearson correlations were examined between each gene and the summed score from both DRRI subscales (Combat Experience Scale and Post-Battle Experience Scale), first within the PTSD group, and separately within the comparison group, in order to identify genes whose expression level varied with the degree of combat stress exposure. Family-wise Bonferonni and FDR q-values were used to correct for multiple observations. Among PTSD cases, the genes associated with the 200 most significant correlations were analyzed for biological annotations enrichment using DAVID.

2.5.2. Discovery and replication of gene-based diagnostic predictors

We utilized a machine-learning technique (support vector machine, SVM) to construct, evaluate, optimize, and cross-validate classification algorithms predicting PTSD status based on gene-expression levels at post-deployment for a *training subset of our full sample*. Training ($n=40$) and validation ($n=10$) subsets (distinct from those utilized in Glatt et al. (2013)) were carved from the full sample using pseudo-random selection in order to preserve a similar distributions of diagnostic status, demographic features (age, ancestry), and covariate values (deployment cohort, prior deployments) for both subsets. All analyses for classifier construction were carried out in the training subset and completely independent from the test subset. Using the same panel of factors and covariates described above, 28,536 ANCOVAs were performed; we generated a large list of candidate genes based on a nominally-selected uncorrected $p<0.01$. The probes on this list were then supplied as potential predictors in an SVM, as various model parameters and predictor combinations were evaluated to identify the model with the highest accuracy in identifying cases and comparison subjects based solely on the expression levels of a minimal gene set identified by shrinking centroids after two-level nested 10-fold cross-validation. The top-performing model was then deployed on the test subset (5 cases and 5 comparison subjects) to determine its generalizability in accurately predicting case status based on gene-expression levels. (The 10 subjects used for model validation were not significantly different from those in the training set in terms of demographic, gene-expression QC, experiential, or clinical factors; data not shown.)

2.5.3. Identification of differentially expressed exons and their associated biological terms

Within the full sample ($n=50$), we examined exon-expression levels utilizing 22,204 ANCOVAs to identify putative alternative splicing differences between individuals with PTSD and comparison subjects. The same factors evaluated in gene-based analyses (PTSD status, cohort, age, ancestry and prior deployment status) were assessed for their main effects and their interaction with exonID as predictors of exon-expression levels; the PTSD status × exonID interaction term was examined as an indicator of putative alternative splicing, *cf.* (Glatt et al., 2009). Family-wise Bonferroni-correction was applied to the ANCOVA p-values to determine whether any interaction term reached a genome-wide level of significance. We utilized an uncorrected type-I-error rate of 0.0005 to obtain a candidate gene-list for functional profiling and classifier construction. Enrichment analyses were performed using the DAVID algorithm and were evaluated against a Bonferroni-corrected p-value accounting for the number of terms evaluated.

2.5.4. Discovery and replication of exon-based diagnostic predictors

As outlined above for full-length transcripts under Section 2, we used SVMs to construct, evaluate, optimize, and cross-validate classification algorithms predicting eventual PTSD status based on exon-expression levels at pre-deployment for the same *training subset of our full sample*. Using identical subject allocations to training and validation subsets; we first generated a large candidate list of putatively alternatively spliced genes within the training subset (nominal uncorrected $p<0.0005$ for the interaction of PTSD status and exonID), using 22,204 ANCOVAs and the same panel of factors, covariates, and interaction terms described above. For each gene on the list, the most significantly dysregulated exon was identified and supplied as a potential predictor in the SVM classifiers. Various model parameters and predictor combinations were then evaluated to identify the model with the highest accuracy in identifying cases and comparison subjects based solely on the expression levels of a minimal exon set identified by shrinking centroids after two-level nested 10-fold cross-validation. The top-performing model was then deployed on the test subset (5 cases and 5 comparison subjects) to determine its generalizability in accurately predicting case status based on exon-expression levels.

2.6. Validation of gene-expression with quantitative real time polymerase chain reaction

A subset of nine transcripts featured in SVM classifiers were selected for validation with quantitative real time polymerase chain reaction (QRTPCR). First, total RNA was quantitatively converted with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, San Diego City, CA) to generate single-stranded cDNA (for a 20 μl reaction). Aliquots of 20 ng of cDNA were analyzed *via* QRTPCR using

the Prism 7900 HT Fast Real-Time PCR system (Applied Biosystems). Statistical analysis was performed using the comparative ΔCT method. All reactions were run in duplicate and normalized against gyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). For one transcript (*GSTM1*), QRTPCR analysis was repeated with 75 ng cDNA in order to compensate for low signal. The fold change values were compared using independent samples *t*-tests ($p < 0.05$).

3. Results

3.1. Identification of differentially expressed genes and their associated biological terms

No gene's expression level was related to PTSD status at a Bonferroni-corrected level of significance, which is not surprising given the relatively small sample size and large number of transcripts tested. We did, however, identify 64 probes dysregulated with a nominally significant $p < 0.01$ in Marines diagnosed with PTSD (Table 2). Thirty-three of these 64 probes were down-regulated, whereas 31 were up-regulated. Log2 fold-change (FC) of these probes in eventual PTSD cases ranged from 2.00-fold down-regulation to 1.66-fold up-regulation. Among the 64 probes, 59 were recognized pathway participants within the DAVID database; however, no significantly enriched annotations were identified. Exploratory pathway analysis of the differentially expressed genes in Table 2 using the Reactome database also revealed no significant enrichments. When examining gene-expression levels significantly correlated with summed DRRI score, no correlation *p*-values survived genome-wide correction among comparison subjects. Among PTSD cases, 13,336 correlation *p*-values survived an FDR correction threshold of 5%. The probesets featured in the 200 most significant correlations were submitted to DAVID for annotation enrichment analysis. The following terms were significantly enriched, with corresponding Bonferroni-corrected *p*-values: regulation of actin cytoskeleton (5 of 8 probesets down-regulated in PTSD, $p = 0.03$), nucleotide-binding (17 of 30 probesets down-regulated, $p = 0.04$), host-virus interaction (6 of 10 probesets down-regulated, $p = 0.07$), and long-term depression (4 of 5 up-regulated in PTSD, $p = 0.08$).

3.2. Discovery and replication of a gene-based diagnostic predictor

To construct a gene-based classifier and assess its generalizability, we first derived a list of potential classifier transcripts as those probes with a difference in expression between PTSD case and comparison subjects attaining $p < 0.01$ in a training subsample of 20 cases and 20 comparison subjects while controlling for the same factors and covariates as in analysis 1. This analysis and filtering left 66 probes (Table 3) that were then used to build and optimize SVM classifiers. The optimal SVM (identified through two-level nested 10-fold cross-validation with shrinking centroids, cost = 401, tolerance = 0.001, kernel = radial basis function, and gamma = 0.001) comprised just 2 of the 66 starting probes (Table 3, probes in bold font) and attained 78% accuracy in classifying those individuals with PTSD in the training sample. We then tested the identical 2-gene SVM (with the same parameters, but with no shrinkage or cross-validation) in the remaining test subset (5 cases and 5 comparison subjects), where it yielded 80% accuracy. Among cases, four of five were correctly classified, while four of five comparison subjects were also classified correctly. These values correspond to a sensitivity, specificity, positive predictive value and negative predictive value in the test sample of 80%, 80%, 80%, and 80%, respectively. Expression levels for *GSTM1* and *GSTM2* are shown for PTSD cases and comparison subjects in Fig. 1. QRTPCR analysis demonstrated that *GSTM2* expression was reduced among PTSD cases, but results were less consistent for *GSTM1* (Table 4).

3.3. Identification of differentially expressed exons and their associated biological terms

The interaction of diagnosis and exonID identified putative isoform-expression differences ($p < 0.0005$) in 63 genes, 11 of which attained Bonferroni-corrected significance (Table 5). An example of between-group differences in exon expression for one of these eleven genes (*DYNC1LI1*) is illustrated in Fig. 2, where the PTSD cases have significantly lower levels of expression of a single probe corresponding to the fifth exon; this region corresponds to a retained intron, which could account for this pattern of expression difference. The list of 63 genes was analyzed by the DAVID algorithm and Reactome database (Table 6). DAVID analysis revealed five significantly enriched annotations (armadillo-like helical domain, macromolecule catabolic process, acetylation, modification-dependent protein catabolic process, modification-dependent macromolecule catabolic process). Analysis using the Reactome database revealed a single enriched pathway (class 1 MHC mediated antigen processing and presentation).

3.4. Discovery and replication of an exon-based diagnostic predictor

To construct an exon-based classifier and assess its generalizability we first identified potentially differentially spliced exons within our training subsample of 20 cases and 20 comparison subjects based on the diagnosis-x-exonID interaction term, using a nominal threshold of $p < 0.00005$, while controlling for the same factors and covariates as in the analyses above. For genes displaying more than one dysregulated probe between diagnostic groups, we selected the probe with the most significant between-group difference in expression level based on the *p*-values from planned comparisons. This analysis and filtering yielded 56 exons with expression differences between PTSD cases and comparison subjects (Table 7) that were then used to build and optimize SVM classifiers. The optimal SVM (identified through two-level nested ten-fold cross-validation with shrinking centroids, cost = 401, tolerance = 0.001, kernel = radial basis function, and gamma = 0.001) comprised 20 of the 56 starting probes (Table 7, probes in bold font) and attained 100% accuracy in classifying those individuals in the training sample with PTSD. We then tested the identical 20-exon SVM (with the same parameters, but with no

Table 2 Genes significantly dysregulated ($p < 0.01$) in peripheral blood mononuclear cells from the full sample of PTSD cases at post-deployment and used in predictive SVM classifiers[a].

Transcript cluster ID	Gene symbol	Gene product	Diagnostic group main effect		
			Fold-change in cases	*F*	*p*
7971296	*EPSTI1*	Epithelial stromal interaction 1 (breast)	1.66	7.6	8.6E−03
7921434	*AIM2*	Absent in melanoma 2	1.47	10.4	2.4E−03
8056408	*GALNT3*	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 3 (GalNAc-T3)	1.32	17.7	1.3E−04
7970096	*ING1*	Inhibitor of growth family, member 1	1.28	12.3	1.0E−03
8046861	*ITGAV*	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	1.23	10.3	2.5E−03
8102817	*ELF2*	E74-like factor 2 (ets domain transcription factor)	1.23	8.7	5.2E−03
8124022	*DTNBP1*	Dystrobrevin binding protein 1	1.23	13.3	7.0E−04
8145175	*PDLIM2*	PDZ and LIM domain 2 (mystique)	1.21	9.3	3.9E−03
7953032	*LRTM2*	Leucine-rich repeats and transmembrane domains 2	1.20	8.3	6.1E−03
7905131	*CA14*	Carbonic anhydrase XIV	1.20	11.4	1.5E−03
8044613	*CBWD1*	COBW domain containing 1	1.20	10.7	2.1E−03
8067680	*PRIC285*	Peroxisomal proliferator-activated receptor A interacting complex 285	1.18	7.9	7.5E−03
8161537	*CBWD3*	COBW domain containing 3	1.18	8.3	6.2E−03
8155636	*CBWD3*	COBW domain containing 3	1.16	8.4	5.7E−03
8077099	*SCO2*	SCO cytochrome oxidase deficient homolog 2 (yeast)	1.15	7.5	9.0E−03
8012953	*TRIM16*	Tripartite motif-containing 16	1.15	8.2	6.5E−03
8037355	*ZNF428*	Zinc finger protein 428	1.15	8.4	5.8E−03
8161587	*CBWD3*	COBW domain containing 3	1.15	7.4	9.3E−03
7963157	*RACGAP1*	Rac GTPase activating protein 1	1.15	9.2	4.0E−03
7982868	*CHAC1*	ChaC, cation transport regulator homolog 1 (*E. coli*)	1.15	7.3	9.6E−03
7969638	ENST00000459449	Ncrna:snoRNA chromosome:GRCh37:13:95862598:95862702:1	1.14	9.1	4.3E−03
8053248	C2orf65	Chromosome 2 open reading frame 65	1.14	9.9	3.0E−03
8067773	*ZNF512B*	Zinc finger protein 512B	1.13	10.4	2.4E−03
8074916	*C22orf43*	Chromosome 22 open reading frame 43	1.12	10.7	2.1E−03
8139921	*CALN1*	Calneuron 1	1.11	11.1	1.7E−03
7921492	*IGSF9*	Immunoglobulin superfamily, member 9	1.10	7.3	9.6E−03
7975562	*PAPLN*	Papilin, proteoglycan-like sulfated glycoprotein	1.10	7.8	7.9E−03
7996219	*NDRG4*	NDRG family member 4	1.09	8.7	5.0E−03
7919267	AK125616	cDNA FLJ43628 fis, clone SPLEN2027268.	1.09	8.4	5.8E−03
7919347	AK125616	cDNA FLJ43628 fis, clone SPLEN2027268	1.09	8.4	5.8E−03
8064242	*NCRNA00176*	Non-protein coding RNA 176	1.06	12.1	1.1E−03
8161133	*SPAG8*	Sperm associated antigen 8	−1.07	11.1	1.7E−03
7960434	GENSCAN00000019809	cDNA:Genscan chromosome:GRCh37:12:5141715:5142095:-1	−1.07	8.6	5.4E−03

Table 2 (*Continued*)

Transcript cluster ID	Gene symbol	Gene product	Diagnostic group main effect		
			Fold-change in cases	*F*	*p*
7901967	ENST00000489463	Ncrna_pseudogene:scRNA_pseudogene chromosome:GRCh37:1:64121426:64121718:1	−1.08	8.5	5.7E − 03
7926670	ENST00000430957	cDNA:known chromosome:GRCh37:10:23425901:23426107:1	−1.08	8.6	5.2E − 03
8098167	C4orf39	Chromosome 4 open reading frame 39	−1.08	8.6	5.4E − 03
7937474	*NS3BP*	NS3BP	−1.08	8.2	6.3E − 03
7935690	ENST00000471360	Ncrna_pseudogene:Mt_tRNA_pseudogene chromosome:GRCh37:10:101817589:101817658:-1	−1.08	7.6	8.5E − 03
8090366	*UROC1*	Urocanase domain containing 1	−1.08	7.5	8.8E − 03
8103753	*MORF4*	Mortality factor 4	−1.09	8.0	7.2E − 03
8130939	*DLL1*	Delta-like 1 (*Drosophila*)	−1.09	7.7	8.0E − 03
8019437	*CCDC57*	Coiled-coil domain containing 57	−1.09	8.0	7.0E − 03
7966596	*IQCD*	IQ motif containing D	−1.10	13.5	6.3E − 04
7974695	ENST00000480540	cDNA:pseudogene chromosome:GRCh37:14:59261372:59261747:1	−1.10	8.3	6.0E − 03
8175815	*PNCK*	Pregnancy up-regulated non-ubiquitously expressed CaM kinase	−1.10	9.6	3.3E − 03
8030292	*DKKL1*	Dickkopf-like 1	−1.10	8.5	5.6E − 03
7997533	*OSGIN1*	Oxidative stress induced growth inhibitor 1	−1.11	7.7	8.0E − 03
8067671	*SRMS*	Crc-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites	−1.11	7.6	8.3E − 03
7998929	ENST00000470337	Ncrna_pseudogene:tRNA_pseudogene chromosome:GRCh37:16:3220961:3221031:-1	−1.12	7.3	9.7E − 03
8017361	ENST00000460492	cDNA:pseudogene chromosome:GRCh37:17:60593682:60594128:-1	−1.12	15.2	3.2E − 04
7973900	C14orf19	Immunoglobulin (CD79A) binding protein 1 pseudogene	−1.13	12.8	8.4E − 04
7950321	*UCP3*	Uncoupling protein 3 (mitochondrial, proton carrier)	−1.13	11.0	1.8E − 03
7965838	ENST00000411000	Ncrna:snRNA chromosome:GRCh37:12:102190188:102190280:-1	−1.15	9.1	4.2E − 03
8113413	*NUDT12*	Nudix (nucleoside diphosphate linked moiety X)-type motif 12	−1.16	9.2	4.0E − 03
8175775	*MAGEA1*	Melanoma antigen family A, 1 (directs expression of antigen MZ2-E)	−1.16	7.5	8.8E − 03
7948667	*AHNAK*	AHNAK nucleoprotein	−1.18	7.9	7.3E − 03
7913252	*PINK1*	PTEN induced putative kinase 1	−1.18	7.9	7.5E − 03
8118974	*RPL10A*	Ribosomal protein L10a	−1.19	7.8	7.7E − 03
7979551	*PPP2R5E*	Protein phosphatase 2, regulatory subunit B′, epsilon isoform	−1.20	7.3	9.6E − 03
7972021	*TBC1D4*	TBC1 domain family, member 4	−1.21	8.2	6.4E − 03
8106393	*F2R*	Coagulation factor II (thrombin) receptor	−1.23	9.1	4.3E − 03
7900597	C1orf50	Chromosome 1 open reading frame 50	−1.24	10.5	2.3E − 03
7903753	*GSTM2*	Glutathione S-transferase mu 2 (muscle)	−1.58	16.5	2.0E − 04
7903765	*GSTM1*	Glutathione S-transferase mu 1	−2.00	22.0	2.6E − 05

* Rows are sorted by decreasing fold-change in PTSD cases relative to non-PTSD comparison subjects.

Table 3 Genes significantly dysregulated ($p < 0.01$) in peripheral blood mononuclear cells from a subset of PTSD cases at post-deployment and used in predictive SVM classifiers[a].

Transcript cluster ID[#]	Gene symbol	Gene product	Diagnostic group main effect		
			Fold-change in cases	*F*	*p*
7904853	*GPR89A*	G protein-coupled receptor 89A (GPR89A), transcript variant 1, mRNA.	1.46	9.7	3.8E−03
8095139	*SRD5A3*	Steroid 5 alpha-reductase 3 (SRD5A3), mRNA.	1.44	7.7	8.9E−03
8056408	*GALNT3*	UDP-*N*-acetyl-alpha-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase 3 (GalNAc-T3) (GALNT3), mRNA.	1.40	21.9	4.4E−05
8102817	*ELF2*	E74-like factor 2 (ets domain transcription factor) (ELF2), transcript variant 1, mRNA.	1.30	10.0	3.3E−03
7970096	*ING1*	Inhibitor of growth family, member 1 (ING1), transcript variant 4, mRNA.	1.26	7.9	8.2E−03
7938208	*RBMXL2*	RNA binding motif protein, X-linked-like 2 (RBMXL2), mRNA.	1.26	8.8	5.6E−03
8047784	*ZDBF2*	Zinc finger, DBF-type containing 2 (ZDBF2), mRNA.	1.23	8.3	6.8E−03
8124022	*DTNBP1*	Dystrobrevin binding protein 1 (DTNBP1), transcript variant 2, mRNA.	1.20	7.9	8.0E−03
7961418	ENST00000364606	Ncrna:rRNA chromosome:GRCh37:12:13593818:13593935:-1	1.18	11.6	1.7E−03
8162562	C9orf130	Chromosome 9 open reading frame 130 (C9orf130), transcript variant 2, non-coding RNA.	1.16	9.3	4.5E−03
7969638	ENST00000459449	Ncrna:snoRNA chromosome:GRCh37:13:95862598:95862702:1	1.16	8.6	5.9E−03
7930561	*HABP2*	Hyaluronan binding protein 2 (HABP2), transcript variant 1, mRNA.	1.15	10.0	3.3E−03
8067773	*ZNF512B*	Zinc finger protein 512B (ZNF512B), mRNA.	1.14	8.9	5.3E−03
7999356	AF090898	Clone HQ0149 PRO0149 mRNA, complete cds.	1.13	7.6	9.3E−03
8139921	*CALN1*	Calneuron 1 (CALN1), transcript variant 2, mRNA.	1.10	9.0	5.0E−03
7949668	*CCDC87*	Coiled-coil domain containing 87 (CCDC87), mRNA.	1.10	7.9	8.2E−03
8091239	ENST00000516936	Ncrna:rRNA chromosome:GRCh37:3:142310519:142310633:-1	−1.03	7.5	9.8E−03
7910188	ENST00000365394	Ncrna:rRNA chromosome:GRCh37:1:227748882:227749001:1	−1.04	9.7	3.7E−03
8161133	*SPAG8*	Sperm associated antigen 8 (SPAG8), transcript variant 2, mRNA.	−1.07	9.3	4.4E−03
7925250	*GNG4*	Guanine nucleotide binding protein (G protein), gamma 4 (GNG4), transcript variant 2, mRNA.	−1.08	8.7	5.8E−03
8141423	MIR106B	MicroRNA 106b (MIR106B), microRNA.	−1.08	9.3	4.3E−03
7926670	ENST00000430957	Cdna:known chromosome:GRCh37:10:23425901:23426107:1	−1.08	7.7	9.0E−03
7966596	*IQCD*	IQ motif containing D (IQCD), mRNA.	−1.09	10.1	3.2E−03
7946977	*SAA4*	Serum amyloid A4, constitutive (SAA4), mRNA.	−1.09	8.7	5.8E−03
8017361	ENST00000460492	Cdna:pseudogene chromosome:GRCh37:17:60593682:60594128:-1	−1.10	9.1	4.8E−03
8090366	*UROC1*	Urocanase domain containing 1 (UROC1), transcript variant 1, mRNA.	−1.10	8.5	6.2E−03
8018673	*QRICH2*	Glutamine rich 2 (QRICH2), mRNA.	−1.11	8.2	7.1E−03
7973900	C14orf19	Chromosome 14 open reading frame 19 (C14orf19), non-coding RNA.	−1.11	7.5	9.9E−03
7974695	ENST00000480540	Cdna:pseudogene chromosome:GRCh37:14:59261372:59261747:1	−1.11	9.7	3.7E−03
8019437	*CCDC57*	Coiled-coil domain containing 57 (CCDC57), mRNA.	−1.11	8.5	6.1E−03
8130939	*DLL1*	Delta-like 1 (*Drosophila*) (DLL1), mRNA.	−1.12	9.0	5.0E−03
8146334	ENST00000343867	Cdna:pseudogene chromosome:GRCh37:8:48068735:48069425:1	−1.12	9.2	4.7E−03
7988283	LOC645212	Hypothetical LOC645212 (LOC645212), transcript variant 1, non-coding RNA.	−1.12	8.6	5.9E−03
8029693	*FOSB*	FBJ murine osteosarcoma viral oncogene homolog B (FOSB), transcript variant 1, mRNA.	−1.12	8.4	6.6E−03
8034276	*ZNF653*	Zinc finger protein 653 (ZNF653), mRNA.	−1.13	12.8	1.1E−03
8142997	*PLXNA4*	Plexin A4 (PLXNA4), transcript variant 1, mRNA.	−1.13	8.3	6.7E−03
8012126	*CLDN7*	Claudin 7 (CLDN7), transcript variant 1, mRNA.	−1.13	7.7	9.0E−03

Table 3 (*Continued*)

Transcript cluster ID[#]	Gene symbol	Gene product	Diagnostic group main effect		
			Fold-change in cases	*F*	*p*
8071382	*ZNF74*	Zinc finger protein 74 (ZNF74), transcript variant 1, mRNA.	−1.13	8.5	6.3E − 03
8148607	*GLI4*	GLI family zinc finger 4 (GLI4), mRNA.	−1.14	8.2	7.2E − 03
8099279	*ABLIM2*	Actin binding LIM protein family, member 2 (ABLIM2), transcript variant 1, mRNA.	−1.14	11.7	1.7E − 03
8070744	*C21orf2*	Chromosome 21 open reading frame 2 (C21orf2), mRNA.	−1.14	8.3	6.8E − 03
8125149	*SLC44A4*	Solute carrier family 44, member 4 (SLC44A4), transcript variant 1, mRNA.	−1.15	7.5	9.7E − 03
8178653	*SLC44A4*	Solute carrier family 44, member 4 (SLC44A4), transcript variant 1, mRNA.	−1.15	7.5	9.7E − 03
8179861	*SLC44A4*	Solute carrier family 44, member 4 (SLC44A4), transcript variant 1, mRNA.	−1.15	7.5	9.7E − 03
7928306	ENST00000363300	Ncrna:misc_RNA chromosome:GRCh37:10:73980510:73980610:1	−1.15	9.4	4.3E − 03
8141795	*POLR2J3*	Polymerase (RNA) II (DNA directed) polypeptide J3 (POLR2J3), mRNA.	−1.16	8.0	7.9E − 03
8010629	*CCDC137*	Coiled-coil domain containing 137 (CCDC137), mRNA.	−1.16	14.6	5.3E − 04
8112159	*ANKRD55*	Ankyrin repeat domain 55 (ANKRD55), mRNA.	−1.16	9.4	4.2E − 03
8064014	*SLC17A9*	Solute carrier family 17, member 9 (SLC17A9), mRNA.	−1.16	12.1	1.4E − 03
8164665	*RAPGEF1*	Rap guanine nucleotide exchange factor (GEF) 1 (RAPGEF1), transcript variant 1, mRNA.	−1.16	8.6	6.0E − 03
8113413	*NUDT12*	Nudix (nucleoside diphosphate linked moiety X)-type motif 12 (NUDT12), mRNA.	−1.17	7.9	8.3E − 03
7948995	*ATL3*	Atlastin-3 gene:ENSG00000184743	−1.17	8.0	7.9E − 03
8130867	*THBS2*	Thrombospondin 2 (THBS2), mRNA.	−1.17	8.3	6.9E − 03
8010848	*TBCD*	Tubulin folding cofactor D (TBCD), mRNA.	−1.18	7.4	1.0E − 02
7965838	ENST00000363300	Ncrna:snRNA chromosome:GRCh37:12:102190188:102190280:-1	−1.18	10.9	2.3E − 03
8157804	*OLFML2A*	Olfactomedin-like 2A (OLFML2A), mRNA.	−1.19	8.3	6.8E − 03
8118974	*RPL10A*	Ribosomal protein L10a (RPL10A), mRNA.	−1.20	8.8	5.4E − 03
7972021	*TBC1D4*	TBC1 domain family, member 4 (TBC1D4), mRNA.	−1.26	8.1	7.4E − 03
7898679	*NBPF3*	Neuroblastoma breakpoint family, member 3 (NBPF3), mRNA.	−1.27	12.4	1.3E − 03
8088458	*FHIT*	Fragile histidine triad gene (FHIT), transcript variant 1, mRNA.	−1.27	7.7	8.8E − 03
8135268	*EIF4B*	Eukaryotic translation initiation factor 4B (EIF4B), mRNA.	−1.30	8.0	7.7E − 03
7900597	C1orf50	Chromosome 1 open reading frame 50, mRNA (cDNA clone MGC:2448 IMAGE:2959109), complete cds.	−1.31	14.8	4.9E − 04
8138950	*RP9*	Retinitis pigmentosa 9 (autosomal dominant) (RP9), mRNA.	−1.33	9.1	4.8E − 03
8137464	*PSPH*	Phosphoserine phosphatase (PSPH), mRNA.	−1.35	7.8	8.4E − 03
7903753	*GSTM2*	Glutathione S-transferase mu 2 (muscle) (GSTM2), transcript variant 1, mRNA.	−1.51	12.9	1.0E − 03
7903765	*GSTM1*	Glutathione S-transferase mu 1 (GSTM1), transcript variant 1, mRNA.	−2.14	19.7	9.0E − 05

[*] Rows are sorted by decreasing fold-change in PTSD cases relative to non-PTSD comparison subjects.
[#] Transcripts in bold comprised the optimal 2-probe SVM classifier of PTSD status identified by training and testing in independent samples.

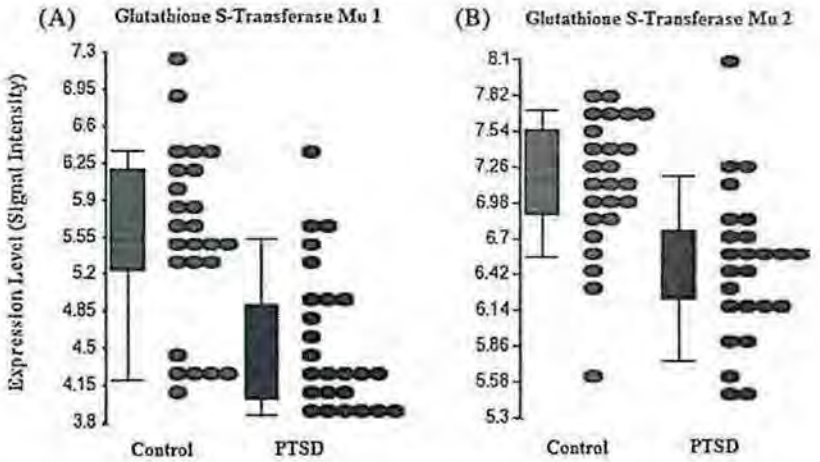

Fig. 1 Microarray-derived expression levels (ordinate) of summarized exon probesets reflecting whole-transcript expression levels (abscissa) of glutathione S-transferase mu 1 (*GSTM1*) and glutathione S-transferase mu 2 (*GSTM2*) in peripheral blood mononuclear cells from PTSD cases ($n = 25$) and comparison subjects ($n = 25$). These transcripts were notably down-regulated among PTSD cases within the full sample (fold changes −1.58 and −2.00, respectively) and were identified as the sole components of the optimal performing SVM classifier of diagnostic status, which achieved 80% accuracy in the test subset ($n = 10$; 4 of 5 cases correctly identified).

shrinkage or cross-validation) in the remaining test subset ($n = 10$; 5 cases and 5 comparison subjects), where it yielded a diminished but reasonable 90% accuracy (higher than the accuracy observed in gene-based analyses). All PTSD cases were correctly classified, while four of five comparison subjects were classified correctly. These values correspond to sensitivity, specificity, positive predictive and negative predictive values of 100%, 80%, 83% and 100%, respectively. QRTPCR analysis of seven exons in the classifier failed to detect significant differences in expression levels between PTSD cases and comparison subjects (Table 4).

4. Discussion

There is emerging support for the hypothesis that peripheral blood transcriptomic signatures associated with PTSD involve dysregulation of genes that function in immune and inflammatory processes or their regulation To this picture we add new and compelling pilot data suggesting that dysregulation of genes whose proteins function in the management of cellular oxidative stress may also be clinically useful biomarkers for distinguishing PTSD cases from trauma-exposed subjects who are resilient to PTSD.

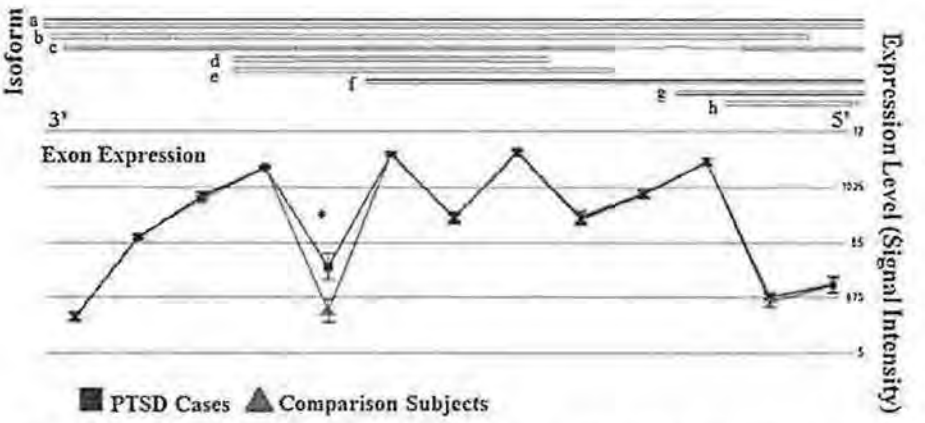

Fig. 2 Microarray-derived expression levels (ordinate) of individual exon-probes (abscissa) of dynein, cytoplasmic 1, light intermediate chain 1 (*DYNC1LI1*) in peripheral blood mononuclear cells from PTSD cases ($n = 25$, squares) and comparison subjects ($n = 25$, triangles). The interaction of diagnosis and exon ID was highly significant ($p = 6.7E-07$, Bonferroni-corrected $p = 1.4E-02$) owing to the selective down-regulated of an exon (probeset ID 8086013; $p = 0.019$) in the context of comparable expression levels of all other exons.

Table 4 QRT-PCR validation of gene and exon expression in full sample of PTSD cases and comparison subjects.

Assay ID	Gene	*GADPH*-normalized average $2^{-\Delta Ct}$		*GADPH*-normalized *p*-values	*HPRT1*-normalized average $2^{-\Delta Ct}$		*HPRT1*-normalized *p*-values
		PTSD	Control		PTSD	Control	
Hs01683722_gH	*GSTM1*	0.367 ± 0.741	1.138 ± 1.529	0.031*	0.685 ± 1.367	1.251 ± 1.139	0.128
Hs03044640_gH	*GSTM2*	0.732 ± 0.596	1.114 ± 0.655	0.036*	0.300 ± 0.184	0.531 ± 0.262	0.001*
Hs00180203_m1	*CUL2*	1.000 ± 0.323	1.223 ± 0.612	0.114	0.768 ± 0.250	0.872 ± 0.350	0.236
Hs00211676_m1	*DYNC1LI1*	0.277 ± 0.261	0.333 ± 0.204	0.401	0.441 ± 0.434	0.466 ± 0.243	0.803
Hs00948075_m1	*HUWE1*	0.333 ± 0.204	0.586 ± 0.570	0.192	0.144 ± 0.105	0.208 ± 0.215	0.184
Hs00277883_m1	*LARP7*	0.674 ± 0.400	0.711 ± 0.272	0.709	0.689 ± 0. 381	0.752 ± 0.331	0.537
Hs00382272_m1	*PNPLA8*	1.115 ± 0.240	1.123 ± 0.276	0.910	1.520 ± 0.694	1.254 ± 0.478	0.122
Hs01554570_m1	*RBM5*	0.462 ± 0.317	0.617 ± 0.621	0.271	0.268 ± 0.183	0.329 ± 0.303	0.390
Hs00208869_m1	*TRAPPC8*	0.788 ± 0.536	0.787 ± 0.375	0.990	0.548 ± 0.372	0.612 ± 0.313	0.512

Values are reported as mean ± standard deviation. *p*-Values < 0.05 are indicated with*.

Yet, dysregulation of genes with immune-, inflammatory- and antioxidant-activity is probably only a small piece of the biological puzzle of PTSD pathophysiology, as many of the differentially expressed genes, as well as the exons comprising the best-performing PTSD-diagnostic classifier, were apparently unrelated to these functions; these other genes had highly disparate functions. Collectively, profiles of dysregulated genes in immune, inflammatory and other pathways may serve as potent biological indicators upon which diagnosis and early intervention may ultimately be based. The present study demonstrates proof-of-principle for the construction of blood-based PTSD diagnostic biomarkers that ranged in accuracy from 80 to 90% in a small subset that was held completely independent from classifier construction.

It is important to note that these classifiers employed decision-rules based solely on mRNA expression levels. To our knowledge, our group is among the first to employ data-driven (SVM) modeling on a list of differentially expressed transcripts in order to identify a subset of transcripts that were most predictive of PTSD status. These two strategies may be useful for identifying exons, genes, and pathways that play a role in the etiology of PTSD, but that may have been overlooked by other approaches focusing on well-established candidate genes. If these profiles of mRNA-expression differences in PTSD cases can be further refined and replicated, and if SVM-based models are found to perform reliably in larger or more diverse populations, then this study proposes an avenue for early diagnosis among trauma-exposed individuals, potentially fostering earlier intervention. However, it is likely that a more accurate classification model can be constructed in the future by taking into account additional known risk factors for PTSD, such as family history, personality traits, pre-existing mental disorders (Koenen et al., 2003a,b), peri-traumatic dissociation and post-trauma social support (Brewin et al., 2000; Ozer et al., 2003), non-genomic biological markers available in the MRS dataset (Baker et al., 2012b; Eraly et al., 2014), and other factors not necessarily related to gene-expression.

The present study did not account for many of these pre- and peri-traumatic risk factors, but future efforts to construct diagnostic models should seek to incorporate such data. Nevertheless, a single diagnostic classifier of PTSD (no matter how precisely constructed) may never perform with 100% accuracy, which is why it will be essential to pursue (in larger samples) the characteristics of subjects for whom such a classifier does not work. Of equal interest is the possibility that there are two or more unique biomarker profiles that are diagnostic of the same phenotypic outcome. In fact, etiologic heterogeneity may be a hallmark of complex disorders including PTSD, so it may not be possible to identify a single "one-size-fits-all" biomarker profile. In the future, methodologies that facilitate the identification of distinct biomarker profiles associated with the same phenotype may be required in order to account for etiologic heterogeneity in PTSD and other complex disorders. Another distinct possibility is that for some cases of PTSD there is no blood-based biomarker signature to be found. We are currently investigating each of these possibilities further. It is also important to acknowledge that the present study did not account for possible effects of pharmacological therapy (*e.g.*, anti-depressants, anxiolytics, and antipsychotics) or

Table 5 Exons significantly dysregulated in peripheral blood mononuclear cells from the full sample of eventual PTSD cases at post-deployment.

Transcript cluster ID	Gene symbol	Gene product	Accession number	*F*	*p*	Adjusted *p*	*q*	Probesets (*n*)	Dysregulated probesets (*n*)	Dysregulated probeset ID*
7903765	*GSTM1*	Glutathione S-transferase mu 1	NM_000561	7.8	1.1E−09	2.3E−05	2.3E−05	9	9	7903767
7954810	*LRRK2*	Leucine-rich repeat kinase 2	NM_198578	2.6	2.8E−09	5.8E−05	2.9E−05	53	10	7954831
8158597	*GPR107*	G protein-coupled receptor 107	NM_001136557	3.1	2.2E−07	4.4E−03	1.5E−03	27	5	8158617
8052443	*USP34*	Ubiquitin specific peptidase 34	NM_014709	2.0	3.9E−07	7.9E−03	2.0E−03	80	4	8052505
8022767	*TRAPPC8*	Trafficking protein particle complex 8	NM_014939	2.8	5.9E−07	1.2E−02	2.3E−03	31	2	8022775
8086008	*DYNC1LI1*	Dynein, cytoplasmic 1, light intermediate chain 1	NM_016141	4.5	6.7E−07	1.4E−02	2.3E−03	13	1	8086013
8136662	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	NM_004668	2.4	9.3E−07	1.9E−02	2.3E−03	46	1	8136700
8070467	*TMPRSS2*	Transmembrane protease, serine 2	NM_001135099	4.2	9.6E−07	1.9E−02	2.3E−03	14	1	8070472
8059596	*TRIP12*	Thyroid hormone receptor interactor 12	NM_004238	2.4	1.0E−06	2.0E−02	2.3E−03	43	2	8059600
8142307	*PNPLA8*	Patatin-like phospholipase domain containing 8	NM_015723	3.9	1.1E−06	2.3E−02	2.3E−03	16	1	8142322
7978285	*ADCY4*	Adenylate cyclase 4	NM_001198592	2.9	2.1E−06	4.3E−02	4.0E−03	26	2	7978294
8149986	*ZNF395*	Zinc finger protein 395	NM_018660	3.9	2.6E−06	5.2E−02	4.3E−03	15	1	8149998
8058118	*KCTD18*	Potassium channel tetramerisation domain containing 18	NM_152387	5.5	5.0E−06	0.10	7.8E−03	8	1	8058121
8010454	*RNF213*	Ring finger protein 213	NM_020914	2.2	1.0E−05	0.21	1.5E−02	44	1	8010469
7946610	*EIF4G2*	Eukaryotic translation initiation factor 4 gamma, 2	NM_001418	2.8	1.5E−05	0.31	2.1E−02	24	1	7946612
7982904	*RTF1*	Rtf1, Paf1	NM_015138	2.8	2.6E−05	0.52	3.3E−02	22	3	7982911
8079869	*RBM5*	RNA binding motif protein 5	NM_005778	2.6	2.9E−05	0.58	3.4E−02	26	1	8079878
8076077	*DDX17*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	NM_006386	3.2	4.1E−05	0.84	4.5E−02	16	3	8076093
7968128	*PABPC3*	Poly(A) binding protein, cytoplasmic 3	NM_030979	11.3	4.2E−05	0.85	4.5E−02	3	1	7968129
8172914	*HUWE1*	HECT, UBA and WWE domain containing 1	NM_031407	1.7	5.0E−05	1.00	5.0E−02	90	5	8172940
7903777	*GSTM5*	Glutathione S-transferase mu 5	NM_000851	4.7	5.2E−05	1.00	5.0E−02	8	3	7903782
8139896	*PMS2P4*	Postmeiotic segregation increased 2 pseudogene 4	NR_022007	8.0	6.3E−05	1.00	5.8E−02	4	1	8139900

Table 5 (*Continued*)

Transcript cluster ID	Gene symbol	Gene product	Accession number	*F*	*p*	Adjusted *p*	*q*	Probesets (*n*)	Dysregulated probesets (*n*)	Dysregulated probeset ID*
8076455	*RRP7A*	Ribosomal RNA processing 7 homolog A (S. cerevisiae)	NM_015703	6.3	9.0E – 05	1.00	7.5E – 02	5	1	8076458
7996677	*NUTF2*	Nuclear transport factor 2	NM_005796	4.9	1.0E – 04	1.00	7.5E – 02	7	2	7996683
8015642	*PSMC3IP*	PSMC3 interacting protein	NM_016556	3.5	1.0E – 04	1.00	7.5E – 02	12	1	8015646
8058182	*FAM126B*	Family with sequence similarity 126, member B	NM_173822	3.5	1.0E – 04	1.00	7.5E – 02	12	0	-
8029884	*SAE1*	SUMO1 activating enzyme subunit 1	NR_027280	4.1	1.1E – 04	1.00	7.5E – 02	9	1	8029892
8096938	*LARP7*	La ribonucleoprotein domain family, member 7	NM_016648	3.0	1.1E – 04	1.00	7.5E – 02	16	2	8096944
7965359	*ATP2B1*	ATPase, Ca^{++} transporting, plasma membrane 1	NM_001001323	2.5	1.1E – 04	1.00	7.5E – 02	24	1	7965379
8159984	C9orf46	Chromosome 9 open reading frame 46	NM_018465	4.8	1.1E – 04	1.00	7.5E – 02	7	1	8159991
7965652	*CDK17*	Cyclin-dependent kinase 17	NM_002595	2.8	1.2E – 04	1.00	7.6E – 02	18	1	7965654
8077858	*ATG7*	ATG7 autophagy related 7 homolog (S. cerevisiae)	NM_006395	2.8	1.2E – 04	1.00	7.6E – 02	18	1	8077874
7971602	*RCBTB1*	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1	NM_018191	3.4	1.2E – 04	1.00	7.6E – 02	12	1	7971613
7971620	*KPNA3*	Karyopherin alpha 3 (importin alpha 4)	NM_002267	2.7	1.3E – 04	1.00	7.8E – 02	19	1	7971637
7956910	*CAND1*	Cullin-associated and neddylation-dissociated 1	NM_018448	2.7	1.4E – 04	1.00	7.8E – 02	19	3	7956914
8002778	*MLKL*	Mixed lineage kinase domain-like	NM_152649	3.3	1.4E – 04	1.00	7.8E – 02	13	1	8002781
8160213	*TTC39B*	Tetratricopeptide repeat domain 39B	NM_152574	2.6	1.4E – 04	1.00	7.8E – 02	22	2	8160226
7962112	*CAPRIN2*	Caprin family member 2	NM_001002259	2.5	1.5E – 04	1.00	7.8E – 02	24	1	7962123
7981068	*SERPINA1*	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	NM_001002236	4.0	1.6E – 04	1.00	8.1E – 02	9	2	7981074
8103951	*ACSL1*	Acyl-CoA synthetase long-chain family member 1	NM_001995	2.5	1.8E – 04	1.00	9.3E – 02	23	1	8103965
7999841	*SMG1*	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (*C. elegans*)	NM_015092	2.0	2.0E – 04	1.00	1.0E – 01	42	4	7999869

Table 5 (*Continued*)

Transcript cluster ID	Gene symbol	Gene product	Accession number	*F*	*p*	Adjusted *p*	*q*	Probesets (*n*)	Dysregulated probesets (*n*)	Dysregulated probeset ID*
7933047	*CUL2*	Cullin 2	NM_003591	2.5	2.1E−04	1.00	0.10	23	2	7933056
8017162	*RNFT1*	Ring finger protein, transmembrane 1	NM_016125	3.6	2.2E−04	1.00	0.10	10	3	8017163
7967881	*MPHOSPH8*	M-phase phosphoprotein 8	NM_017520	3.0	2.4E−04	1.00	0.11	14	1	7967895
7900426	*SMAP2*	Small ArfGAP2	NM_022733	3.4	2.5E−04	1.00	0.11	11	1	7900432
7903893	*CD53*	CD53 molecule	NM_000560	3.3	2.5E−04	1.00	0.11	12	2	7903894
8078569	*GOLGA4*	Golgin A4	NM_002078	2.2	2.6E−04	1.00	0.11	30	2	8078594
7967117	*OASL*	2′—5′-oligoadenylate synthetase-like	NM_003733	3.8	2.6E−04	1.00	0.11	9	3	7967123
7975416	*PCNX*	Pecanex homolog (*Drosophila*)	NM_014982	2.1	2.7E−04	1.00	0.11	36	1	7975429
8179298	*CSNK2B*	Casein kinase 2, beta polypeptide	NM_001320	3.6	2.8E−04	1.00	0.11	10	1	8179308
8143327	*PARP12*	Poly (ADP-ribose) polymerase family, member 12	NM_022750	3.1	2.9E−04	1.00	0.11	13	2	8143336
8021496	KIAA1468	KIAA1468	NM_020854	2.2	2.9E−04	1.00	0.11	31	4	8021504
8141846	*FBXL13*	F-box and leucine-rich repeat protein 13	NM_145032	2.3	3.0E−04	1.00	0.12	25	4	8141865
7929677	*PI4K2A*	Phosphatidylinositol 4-kinase type 2 alpha	NM_018425	3.3	3.2E−04	1.00	0.12	11	0	-
8088348	*FAM116A*	Family with sequence similarity 116, member A	NM_152678	2.5	3.3E−04	1.00	0.12	20	2	8088366
7937363	*PKP3*	Plakophilin 3	NM_007183	3.0	3.5E−04	1.00	0.13	14	2	7937370
8089785	*POPDC2*	Popeye domain containing 2	NM_022135	3.7	3.6E−04	1.00	0.13	9	2	8089794
7967563	*UBC*	Ubiquitin C	NM_021009	2.9	3.6E−04	1.00	0.13	14	2	7967584
8077171	*RABL2B*	RAB, member of RAS oncogene family-like 2B	NM_001130921	3.0	3.7E−04	1.00	0.13	13	1	8077180
8112772	*AP3B1*	Adaptor-related protein complex 3, beta 1 subunit	NM_003664	2.2	3.7E−04	1.00	0.13	28	2	8112794
7997626	*KLHL36*	Kelch-like 36 (*Drosophila*)	NM_024731	4.7	3.9E−04	1.00	0.13	6	2	7997629
8043251	*PTCD3*	Pentatricopeptide repeat domain 3	NM_017952	2.3	5.0E−04	1.00	0.16	24	0	-
8072170	*KREMEN1*	Kringle containing transmembrane protein	NM_032045	2.9	5.0E−04	1.00	0.16	14	4	8072173

* Rows are sorted by increasing *p*-value for the interaction of diagnosis and exonID.

* Exon probesets listed were the most significantly differentially expressed (per gene) between diagnostic groups. Information on the identities of all dysregulated exons for each gene can be obtained from the authors upon request.

Table 6 Annotations enriched at corrected significance levels among differentially-expressed exons ($p < 0.0005$) in peripheral blood mononuclear cells from the full sample of PTSD cases at post-deployment*.

DAVID category	Term	Count (%)	Fold-enrichment	*p*	Bonferroni-corrected *p*	Gene corresponding to dysregulated exon
INTERPRO	IPR011989:armadillo-like helical	7 (11.1%)	14.9	6.5E – 06	1.0E – 03	*KIAA1468, AP3B1, PKP3, CAND1, TRIP12, KPNA3, LRRK2*
GOTERM_BP_FAT	GO:0009057 ~ macromolecule catabolic process	12 (19.0%)	4.5	4.2E – 05	1.8E – 02	*SAE1, SMG1, USP34, FBXL13, HUWE1, CAND1, ATG7, TRIP12, MGAM, CUL2, KLHL36, UBC*
SP_PIR_KEYWORDS	Acetylation	22 (34.9%)	2.5	5.8E – 05	7.9E – 03	*EIF4G2, AP3B1, NUTF2, SMG1, HUWE1, PTCD3, CUL2, LARP7, UBC, ACSL1, KIAA1468, CSNK2B, SAE1, PABPC3, RTF1, ATG7, CAND1, KPNA3, RNF213, RBM5, MPHOSPH8, GSTM5*
GOTERM_BP_FAT	GO:0019941 ~ modification-dependent protein catabolic process	10 (15.9%)	5.1	1.0E – 04	4.3E – 02	*SAE1, USP34, FBXL13, HUWE1, CAND1, ATG7, TRIP12, CUL2, KLHL36, UBC*
GOTERM_BP_FAT	GO:0043632 ~ modification-dependent macromolecule catabolic process	10 (15.9%)	5.1	1.0E – 04	4.3E – 02	*SAE1, USP34, FBXL13, HUWE1, CAND1, ATG7, TRIP12, CUL2, KLHL36, UBC*
Reactome	Term	Count (%)		*p*	FDR-corrected *p*	Gene corresponding to dysregulated exon
	Class 1 MHC mediated antigen processing and presentation	6 (9.9%)		1.0E – 04	1.5E – 02	*SAE1, CUL2, ATG7, TRIP12, HUWE1, UBC*

* Rows are sorted by increasing *p*-value for the enrichment of annotations.

Table 7 Exons Significantly Dysregulated in Peripheral Blood Mononuclear Cells from a Subset of PTSD Cases at Post-Deployment and Used in Predictive SVM Classifiers*.

Transcript Cluster ID#	Gene Symbol	Gene Product	Interaction *p*	Exon ID	Fold-change	*F*	*p*
8096938	***LARP7***	**La ribonucleoprotein domain family, member 7**	**7.2E – 09**	**8096944**	**3.52**	**8.6**	**5.9E – 03**
8097148	***RNF213***	**Ring finger protein 213**	**7.1E – 11**	**8010469**	**3.06**	**9.6**	**3.8E – 03**
8086008	***DYNC1LI1***	**Dynein, cytoplasmic 1, light intermediate chain 1**	**4.0E – 10**	**8086013**	**3.06**	**7.9**	**8.3E – 03**
8134122	***PTCD3***	**Pentatricopeptide repeat domain 3**	**1.6E – 07**	**8043256**	**3.05**	**7.7**	**9.0E – 03**
8142307	***PNPLA8***	**Patatin-like phospholipase domain containing 8**	**5.3E – 09**	**8142322**	**2.87**	**9.3**	**4.4E – 03**
8172914	***DIDO1***	**Death inducer-obliterator 1**	**3.7E – 06**	**8067576**	**2.77**	**5.1**	**3.0E – 02**
8076455	***RRP7A***	**Ribosomal RNA processing 7 homolog A (S.cerevisiae)**	**1.7E – 05**	**8076458**	**2.74**	**12.8**	**1.1E – 03**
8158597	***GPR107***	**G protein-coupled receptor 107**	**2.3E – 10**	**8158617**	**2.74**	**11.5**	**1.8E – 03**
8054092	***CUL2***	**Cullin 2**	**3.4E – 06**	**7933056**	**2.71**	**11.9**	**1.5E – 03**
8105191	**KIAA1468**	**KIAA1468**	**1.0E – '07**	**8021504**	**2.71**	**11.1**	**2.1E – 03**
8045090	*ZC3H11A*	Zinc finger CCCH-type containing 11A	1.1E – 05	7908985	2.67	6.5	1.6E – 02
8056837	***TTC17***	**Tetratricopeptide repeat domain 17**	**1.1E – 06**	**7939453**	**2.63**	**6.3**	**1.7E – 02**
8079869	***NEK9***	**NIMA (never in mitosis gene a)-related kinase 9**	**6.7E – 06**	**7980282**	**2.62**	**8.4**	**6.4E – 03**
8079869	***RBM5***	**RNA binding motif protein 5**	**1.5E – 08**	**8079878**	**2.62**	**8.9**	**5.3E – 03**
8105191	*PARP8*	Poly (ADP-ribose) polymerase family, member 8	1.6E – 05	8105199	2.57	4.6	4.0E – 02
8083523	***AQR***	**Aquarius homolog (mouse)**	**2.6E – 05**	**7987328**	**2.57**	**6.3**	**1.7E – 02**
8136662	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	5.1E – 08	8136700	2.57	4.4	4.3E – 02
8107375	***TRAPPC8***	**Trafficking protein particle complex 8**	**6.8E – 08**	**8022775**	**2.56**	**7.1**	**1.2E – 02**
8136662	***UGGT1***	**UDP-glucose glycoprotein glucosyltransferase 1**	**2.1E – 06**	**8045104**	**2.53**	**10.7**	**2.5E – 03**
8169541	*XRN2*	5′–3′ exoribonuclease 2	2.7E – 05	8061333	2.46	6.4	1.6E – 02
8103951	*ACSL1*	Acyl-CoA synthetase long-chain family member 1	1.2E – 05	8103961	2.45	5.2	3.0E – 02
8172914	***HUWE1***	**HECT, UBA and WWE domain containing 1**	**1.3E – 07**	**8172982**	**2.42**	**7.7**	**9.0E – 03**
8061324	*CAND1*	Cullin-associated and neddylation-dissociated 1	5.1E – 06	7956914	2.42	8.1	7.4E – 03
8160213	***TTC39B***	**Tetratricopeptide repeat domain 39B**	**3.3E – 06**	**8160226**	**2.37**	**10.2**	**3.0E – 03**
8107375	*YTHDC2*	YTH domain containing 2	9.5E – 06	8107388	2.35	12.7	1.1E – 03
8149986	*ZNF395*	Zinc finger protein 395	4.6E – 08	8149998	2.32	8.1	7.4E – 03
8076455	*CDK17*	Cyclin-dependent kinase 17	5.8E – 06	7965654	2.25	8.2	7.2E – 03
8149986	*USP34*	Ubiquitin specific peptidase 34	1.5E – 10	8052505	2.20	7.7	9.0E – 03
8092933	*SMG1*	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (*C. elegans*)	5.0E – 07	7994025	2.18	5.0	3.2E – 02
8156321	*TMEM131*	Transmembrane protein 131	2.2E – 05	8054124	2.18	5.6	2.3E – 02
8158597	*GPR155*	G protein-coupled receptor 155	3.6E – 06	8056852	2.15	6.7	1.4E – 02
8086008	*ADAM10*	ADAM metallopeptidase domain 10	7.6E – 06	7989240	2.10	5.1	3.0E – 02
8092933	*ACAP2*	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2	2.4E – 05	8092954	2.10	5.0	3.1E – 02

Table 7 (*Continued*)

Transcript Cluster ID[#,*]	Gene Symbol	Gene Product	Interaction *p*	Exon ID	Fold-change	*F*	*p*
8051882	*DENND2D*	DENN	3.4E − 06	7918493	2.07	5.7	2.3E − 02
8156321	*SYK*	Spleen tyrosine kinase	2.0E − 06	8156330	1.93	13.3	8.7E − 04
8083523	*GMPS*	Guanine monphosphate synthetase	8.9E − 06	8083535	1.83	5.9	2.1E − 02
8134122	*AKAP9*	A kinase (PRKA) anchor protein (yotiao) 9	7.8E − 07	8134144	1.81	8.3	6.9E − 03
8160213	*TRIP12*	Thyroid hormone receptor interactor 12	1.2E − 06	8059619	1.79	6.5	1.6E − 02
8059596	*EIF4G2*	Eukaryotic translation initiation factor 4 gamma, 2	7.3E − 07	7946612	1.76	5.9	2.1E − 02
8130151	*APC2*	Adenomatosis polyposis coli 2	3.6E − 05	8024308	1.75	7.0	1.2E − 02
8130151	*RAET1E*	Retinoic acid early transcript 1E	3.7E − 05	8130160	1.74	8.1	7.4E − 03
8076077	*CAPRIN2*	Caprin family member 2	6.6E − 06	7962123	1.69	6.4	1.6E − 02
8103951	*NUP88*	Nucleoporin 88 kDa	4.9E − 05	8011838	1.68	5.6	2.3E − 02
8078569	*MPHOSPH8*	M-phase phosphoprotein 8	1.9E − 05	7967895	1.63	18.3	1.5E − 04
8052443	*FAM175B*	Family with sequence similarity 175, member B	2.7E − 05	7931218	1.61	6.8	1.3E − 02
8097148	KIAA1109	KIAA1109	1.7E − 05	8097151	1.59	7.7	9.1E − 03
8169541	*DOCK11*	Dedicator of cytokinesis 11	1.4E − 05	8169559	1.41	5.5	2.5E − 02
8022767	*SMAP2*	Small ArfGAP2	3.0E − 05	7900432	−1.12	6.1	1.9E − 02
8179298	*DDX17*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	1.1E − 06	8076093	−1.12	5.6	2.4E − 02
8067563	*MDM2*	Mdm2 p53 binding protein homolog (mouse)	6.3E − 07	7957003	− 1.16	5.5	2.5E − 02
8096938	*SMG1*	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (*C. elegans*)	2.7E − 08	7999869	−1.20	14.1	6.6E − 04
8043251	*GSTM5*	Glutathione S-transferase mu 5	4.2E − 05	7903782	−1.57	18.5	1.4E − 04
8078569	*GOLGA4*	Golgin A4	5.0E − 06	8078597	−1.62	5.2	2.9E − 02
8142307	*LRPPRC*	Leucine-rich PPR-motif containing	5.6E − 07	8051896	−1.77	10.4	2.8E − 03
8179298	*CSNK2B*	Casein kinase 2, beta polypeptide	2.3E − 05	8179308	−1.83	7.8	8.4E − 03
8024306	***GSTM1***	**Glutathione S-transferase mu 1**	**4.5E − 09**	**7903772**	**−2.52**	**21.4**	**5.3E − 05**

* Rows are sorted by decreasing fold-change in eventual PTSD cases relative to non-PTSD comparison subjects.

Exons of Transcript Cluster IDs in bold comprised the optimal 20-probe SVM classifier of eventual PTSD status identified by training and testing in independent samples.

other treatments on post-deployment gene-expression profiles. Five of the 25 PTSD subjects reported using at least one psychiatric medication at the time blood samples were obtained, while none of the comparison subjects reported psychiatric medication use. It is plausible that between-group differences in medication use could account for some of the gene-expression differences observed between these groups. In order to account for this possibility, we performed a separate ANCOVA comparing non-medicated PTSD subjects ($n = 20$) and comparison subjects ($n = 25$). The removal of medicated subjects from the PTSD group produced only minor changes in ANCOVA fold-change values for the genes of interest; the average difference in fold-change value was <2%. Previous genome-wide expression studies have addressed this issue by using samples from PTSD subjects who were not currently medicated (Zieker et al., 2007; Yehuda et al., 2009; Neylan et al., 2011). Other studies have not explicitly address medication status (Mehta et al., 2011; Segman et al., 2005). However, if the ultimate goal is to develop gene-expression-based diagnostic classifiers that are robust to real-world variability, then the inclusion of medicated subjects may be valuable. Future studies should attempt to account for medication status and statistically control for its effect on gene-expression in order to identify genes that are specific to PTSD pathophysiology.

Because of our relatively small sample size and the severe corrections for multiple-testing required when examining the entire transcriptome, we did not detect individual gene-expression differences in PTSD cases that surpassed stringent criteria for declaring statistical significance. As such, the focus of our efforts and interpretations has been on groups of genes, either in regard to their biological annotations or their collective ability to identify PTSD cases. Nevertheless, one gene identified here as dysregulated has

been identified previously in studies seeking to identify blood-based diagnostic biomarkers for PTSD. Prior to rigorous correction for multiple observations, Neylan et al. (2011) reported up-regulation of *GSTM1* in PTSD cases, whereas we observed down-regulation of *GSTM1* in PTSD cases. It is plausible that differences in subject characteristics or study design could account for the discrepant findings. Neylan et al. (2011) found increased *GSTM1* expression in PTSD subjects compared to a non-trauma exposed control group. Perhaps these discrepant findings could make sense in the context of a model where increased *GSTM1* expression reflects an adaptive response to traumatic stress and the attenuation of this response disposes some trauma-exposed individuals to developing PTSD., These studies also differed with respect to the time-span between disease onset and blood sample collection. Remarkably, *GSTM1* and *GSTM2* were identified as the lone predictors within a diagnostic classifier that achieved 80% accuracy in the test subset, and the down-regulation of *GSTM2* was confirmed by QRTPCR. In previous work, we observed down-regulation of *GSTM1* among these same subjects in samples taken prior to their deployment and the development of clinically significant PTSD symptoms; *GSTM1* expression levels were also part of a pre-deployment predictor of subsequent PTSD diagnosis (Glatt et al., 2013). Members of this enzyme class function in the detoxification of electrophilic compounds – including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress – by conjugation with glutathione. Down-regulation of genes whose proteins are responsible for the metabolism of reactive oxygen species (ROS) was also observed in lifetime PTSD cases with current symptoms (Zieker et al., 2007). The apparent link between ROS metabolism and PTSD may make more sense in light of previous *in vitro* studies demonstrating redox regulation of intracellular GR signaling. Specifically, reduced expression of antioxidant protein or direct administration of ROS negatively modulated GR signaling and resulted in reduced expression of glucocorticoid-induced genes; this effect could be rescued by the administration of antioxidant compounds (Makino et al., 1996; Okamoto et al., 1999). It is also interesting to note that other groups have found associations between *GSTM1* polymorphisms and other brain disorders, including schizophrenia (Gravina et al., 2011; Rodriguez-Santiago et al., 2010), bipolar disorder (Mohammadynejad et al., 2011), and alcohol withdrawal symptoms (Okubo et al., 2003).

Despite our relatively small sample size and the additional levels of correction for multiple-testing required for exon analyses, a number of differentially expressed exons surpassed stringent criteria for declaring statistical significance. Additionally, the exon-based predictive classifier appeared to perform better than the gene-based predictive classifier. Taken together, these data support our previous findings (Glatt et al., 2013), suggesting that exon expression (indexing the activity of individual splice variants) may be more reliable and biologically informative than the expression of full-length "genes" or aggregated transcript clusters. Yet, we could not successfully recapitulate these array-derived results by QRTPCR, so further validation attempts must be made. Two of the dysregulated exons we identified by array analysis are components of genes *DDX17* and *FAM175B*, which have been identified as differentially expressed in previous PTSD biomarker studies. Yehuda et al. (2009) observed up-regulation of *DDX17* among PTSD cases. Sarapas et al. (2011) observed down-regulation of *FAM175B* among current PTSD cases, but not lifetime PTSD cases or trauma-exposed comparison subjects. It is also curious to note that the list of alternatively spliced transcripts was enriched for acetylation-dependent protein catabolism and acetylation-regulated proteins more generally. Emerging evidence indicates that the acetylation of amino acids within non-histone proteins plays a role in regulation of cell metabolism (Choudhary et al., 2009; Yang and Seto, 2008). If the differentially expressed exons in PTSD are found to contain acetylation-dependent regulatory domains, then it is plausible that the PTSD proteome may be abnormally (hypo- or hyper-) responsive to acetylation.

It is interesting to compare the results of the present pilot study with our own previous work examining pre-deployment gene-expression profiles associated with subsequent development of PTSD after return from deployment (Glatt et al., 2013), as many of the same subjects (24 of 25 cases, 23 of 25 comparison) were featured in both studies. A number of whole-gene transcripts appeared dysregulated both prior to deployment (in those who would later develop PTSD) and after deployment (in current PTSD cases). *AIM2* and *EPSTI1* were up-regulated in both analyses *(i.e.*, at both pre- and post-deployment), while *RPL10A* and *GSTM1* were down-regulated in both analyses; these may reflect stable markers for PTSD vulnerability. Alternatively, they could have been dysregulated at pre-deployment assessment because pathophysiogical changes had already begun among these subjects, many of whom had previously been deployed to war zones (or because of other unmeasured factors, such as early adversity). *LRTM2*, on the other hand, was down-regulated in pre-deployment samples, but up-regulated in post-deployment samples; this may reflect a maladaptive change or a compensatory yet ultimately ineffective change. Additionally, one putatively alternatively spliced transcript was identified in both pre- and post-deployment analyses. For *MGAM*, a similar pattern of dysregulated exon expression was observed in both analyses, with PTSD cases showing increased expression of several probes and the largest expression difference observed in a probe (8136700) spanning the 23rd and 24th exons. This may also reflect a stable marker for PTSD vulnerability.

The present pilot study broadens the search for diagnostic biomarkers for PTSD beyond that of previous work. Several studies have compared genome-wide transcriptional profiles between PTSD cases and controls, but to our knowledge, very few transcripts have been identified as dysregulated across studies using independent samples. Two studies with overlapping sample pools reported reduced expression of *FKBP5* among current PTSD cases (Sarapas et al., 2011; Yehuda et al., 2009). A third study by Mehta et al. (2011) reported that the interaction of PTSD status and *FKBP5* genotype (rs9296158) was associated with dysregulation of *FKBP5* expression. Pre-deployment expression levels of *FKBP5* were also shown to independently predict post-deployment PTSD symptoms (van Zuiden et al., 2012a). In the present microarray study, diagnostic status was not associated with differences in *FKBP5* expression, suggesting a need to further consider heterogeneity in PTSD etiology. As discussed above, *GSTM1* was originally identified as up-regulated among PTSD

Table 8 Annotations enriched at corrected significance levels among dysregulated transcripts across studies comparing PTSD cases and comparison subjects.

DAVID category	Term	Count (%)	Fold-enrichment	*p*	Bonferroni-corrected (*p*)	Gene corresponding to dysregulated exon
GOTERM_BP_FAT	GO:0001775 ~ cell activation	15 (8.1%)	5.1	1.2E − 06	2.1E − 03	*BLM, SWAP70, STAT5B, KLRK1, MINK1, PF4, IGF2, SLAMF1, TGFB1, AZU1, CCND3, C14ORF19, TREML1, F2R, CD7*
GOTERM_BP_FAT	GO:0009611 ~ response to wounding	19 (10.2%)	3.5	6.6E − 06	1.1E − 02	*LIPA, CCR1, STAT5B, CXCR1, PF4, IGF2, SOD1, CCL5, DTNBP1, TGFB1, GP9, AZU1, ORM1, CX3CR1, CTSB, PLA2G4C, TREML1, KDM6B, F2R*
GOTERM_CC_FAT	GO:0030141 ~ secretory granule	10 (5.4%)	5.7	5.9E − 05	1.3E − 02	*AZU1, SPAG8, PPBP, CPA3, PF4, SPARC, TREML1, RACGAP1, TGFB1, GP9*
GOTERM_BP_FAT	GO:0001817 ~ regulation of cytokine production	11 (5.9%)	6.0	1.5E − 05	2.5E − 02	*AZU1, IL18, TIA1, STAT5B, KLRK1, IGF2, PF4, TRIM16, SOD1, TGFB1, F2R*
GOTERM_CC_FAT	GO:0031091 ~ platelet alpha granule	6 (3.2%)	11.0	2.0E − 04	4.1E − 02	*PPBP, PF4, SPARC, TREML1, TGFB1, GP9*
GOTERM_CC_FAT	GO:0000323 ~ lytic vacuole	10 (5.4%)	4.9	2.0E − 04	4.2E − 02	*AZU1, LAPTM5, LIPA, MMD, IFI30, CPA3, CTSC, CTSB, ASAH1, GBA*
GOTERM_CC_FAT	GO:0005764 ~ lysosome	10 (5.4%)	4.9	2.0E − 04	4.2E − 02	*AZU1, LAPTM5, LIPA, MMD, IFI30, CPA3, CTSC, CTSB, ASAH1, GBA*
KEGG_PATHWAY	hsa04060:cytokine-cytokine receptor interaction	13 (7.0%)	3.2	5.0E − 04	4.5E − 02	*TNFRSF21, IL18R1, IL18, CCR1, CXCR1, PF4, CCL5, TGFB1, PPBP, CX3CR1, CSF2RB, IL2RG, IL3RA*
GOTERM_MF_FAT	GO:0019955 ~ cytokine binding	8 (4.3%)	7.0	1.4E − 04	4.9E − 02	*IL18R1, CCR1, CX3CR1, CSF2RB, CXCR1, IL2RG, TRIM16, IL3RA*
Reactome	**Term**	**Count (%)**		***p***	**FDR-corrected *p***	**Gene corresponding to dysregulated exon**
	Cytokine-cytokine receptor interaction	13 (7.0%)		1.0E − 05	3.0E − 03	*IL18, TGFB1, PPBP, CX3CR1, CCL5, IL3RA, CXCR1, CSF2RB, TNFRSF21, CCR1, PF4, IL2RG, IL18R1*
	Lysosome	7 (3.8%)		3.0E − 04	3.5E − 02	*NAPSA, LAPTM5, ASAH1, CTSC, CTSB, GBA, LIPA*
	Interleukin signaling pathway	5 (2.7%)		3.0E − 04	4.7E − 02	*IL16, IL18, STAT5B, IL3RA, CXCR1*

cases by Neylan et al. (2011), but was found to be down-regulated prior to deployment among US Marines who would later develop PTSD and down-regulated among current PTSD cases within the present analysis, which utilized many of the same subjects as Glatt et al. (2013). Also discussed above, the present study was the second to implicate *DDX17* and *FAM175B* transcripts. The paucity of overlapping findings across studies may be accounted for by a number of factors, including the high risk for Type 1 error inherent when the number of subjects is small and statistical correction for multiple observations is inadequate. Other factors could also contribute to discrepant findings, including differences in trauma exposure (military combat, catastrophic event), sample type (PBMC *vs.* whole blood), the timing of sampling with respect to trauma exposure and disease onset, or differences in PTSD treatment effects across studies.

Comparing annotation-enrichment results across studies provided no additional consensus on the nature of gene-expression dysregulation in PTSD; we performed DAVID and Reactome analyses on individual transcript lists provided by each of the reviewed studies (Mehta et al., 2011; Neylan et al., 2011; Sarapas et al., 2011; van Zuiden et al., 2012a; Yehuda et al., 2009; Zieker et al., 2007), but few studies demonstrated significant enrichment of terms and no common terms were observed across studies. However, when these transcript lists were combined with the present data to create a single list, significant enrichment was observed for a number of terms related to cytokine signaling, lysosomal activity, and other immune-cell activities (Table 8). It is apparent that genes involved in cellular immunity are reliably and disproportionately represented among those that are dysregulated in PTSD cases. This is supported by a large body of evidence for dysfunctional cellular immune processes in individuals with PTSD, which we recently reviewed in depth (Baker et al., 2012b). Our review of the collective evidence suggests that systemic inflammation and deleterious health consequences in PTSD are strongly linked. Given this evidence, treatment strategies to reduce inflammation or modulate cell-mediated immunological processes may be of value to pursue in preclinical models of PTSD.

In conclusion, as the development of PTSD following initial trauma exposure remains quite variable and unpredictable, we sought to identify readily assessable biomarkers to aid in early diagnosis based on evaluations of blood-based gene-expression among Marines participating in the MRS. Our analyses converged on a diverse group of genes and exons that appeared to be differentially expressed in peripheral blood cells from individuals with PTSD. Reduced expression of two genes involved in ROS metabolism were predictive of PTSD diagnostic status, while altered exon expression within a larger and more heterogeneous group of transcripts also predicted the PTSD diagnosis with apparently high accuracy. If blood-based biomarkers (such as the panels of genes and exons identified here) can be validated in additional cohorts exposed to a wider variety of traumatic stressors, then they may serve as useful adjuncts to the prevailing gold-standard behavioral diagnostic systems (Brewin, 2005; Ozer et al., 2003). Enabling clinicians to more confidently diagnose PTSD at earlier stages would be particularly important in groups such as these Marines, for whom it is known in advance that exposure to serious trauma is highly likely. This may also prove highly relevant for first-responders, such as police, fire, and emergency medical teams, for whom a regular part of their job is also exposure to potentially traumatic situations. Furthermore, blood-based biomarkers may help clinicians identify instances of determination of fitness for duty so that support services and limited resources can be made available to those individuals with the greatest need.

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Conflict of interest statement

The authors report no conflicts of interest

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The role of biomarkers and MEG-based imaging markers in the diagnosis of post-traumatic stress disorder and blast-induced mild traumatic brain injury

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Summary
Background: Pervasive use of improvised explosive devices (IEDs), rocket-propelled grenades, and land mines in the recent conflicts in Iraq and Afghanistan has brought traumatic brain injury (TBI) and its impact on health outcomes into public awareness. Blast injuries have been deemed signature wounds of these wars. War-related TBI is not new, having become prevalent during WWI and remaining medically relevant in WWII and beyond. Medicine's past attempts to accurately diagnose and disentangle the pathophysiology of war-related TBI parallels current lines of inquiry and highlights limitations in methodology and attribution of symptom etiology, be it organic, psychological, or behavioral. New approaches and biomarkers are needed.
Preclinical: Serological biomarkers and biomarkers of injury obtained with imaging techniques represent cornerstones in the translation between experimental data and clinical observations. Experimental models for blast related TBI and PTSD can generate critical data on injury threshold, for example for white matter injury from acceleration. Carefully verified and validated models can be evaluated with gene expression arrays and proteomics to identify new candidates for serological biomarkers. Such models can also be analyzed with diffusion MRI and microscopy in order to identify criteria for detection of diffuse white matter injuries, such as DAI (diffuse axonal injury). The experimental models can also be analyzed with focus on injury outcome in brain stem regions, such as locus coeruleus or nucleus raphe magnus that can be involved in response to anxiety changes.

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Clinical: Mild (and some moderate) TBI can be difficult to diagnose because the injuries are often not detectable on conventional MRI or CT. There is accumulating evidence that injured brain tissues in TBI patients generate abnormal low-frequency magnetic activity (ALFMA, peaked at 1–4 Hz) that can be measured and localized by magnetoencephalography (MEG). MEG imaging detects TBI abnormalities at the rates of 87% for the mild TBI, group (blast-induced plus non-blast causes) and 100% for the moderate group. Among the mild TBI patients, the rates of abnormalities are 96% and 77% for the blast and non-blast TBI groups, respectively. There is emerging evidence based on fMRI and MEG studies showing hyper-activity in the amygdala and hypo-activity in pre-frontal cortex in individuals with PTSD. MEG signal may serve as a sensitive imaging marker for mTBI, distinguishable from abnormalities generated in association with PTSD. More work is needed to fully describe physiological mechanisms of post-concussive symptoms.
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1. Background

Blast injuries are deemed the signature wounds of the first wars (Afghanistan and Iraq) of the 21st century (Lancet, 2007; Galarneau et al., 2008). According to a recent U.S. Department Veterans Affairs (DVA) and Defense (DoD) report, 12–23% of returning service members reported a TBI during deployment (O'Neil et al., 2013). Of these, the majority are in the "mild" range of severity (mTBI) (Centers for Disease Control and Prevention and National Center for Injury Prevention and Control 2003; Hoge et al., 2009; O'Neil et al., 2013). A review of the literature from 20th century wars (WWI, WWII, Vietnam) shows that current lines of scientific inquiry regarding the etiology of those symptoms parallel earlier attempts to disentangle the pathophysiology of post-concussive symptoms (PCS) from mental health symptoms, and to distinguish mTBI from war-related mental health syndromes, such as PTSD (Jones et al., 2007). Moreover this literature highlights limitations in methodology and attribution of symptom etiology, be it organic, psychological, or behavioral, that remain a focus of investigations today (Myers, 1915; Fulton, 1942; Jones et al., 2007; Rosenfeld et al., 2013).

A particular challenge in disentangling the symptoms and physiology has been establishing a quantitative, unassailable diagnostic methodology for defining mTBI, such a distinguishing blood or imaging biomarker signature. Most studies have relied on self-report of a concussive event, and have defined mTBI clinically, using symptom-based criteria. Brain changes that may accompany mTBI have been hard to visualize using standard imaging methods (Huang et al., 2012). While neurocognitive tests are used clinically and can be helpful, authors of the recent U.S. DVA Report observed that only a few studies among those reviewed found an association between mTBI and cognitive deficits (O'Neil et al., 2013). However, longitudinal follow-up of military personnel initially evacuated to Longstuhl with mTBI (self-report of war-related brain injury event) showed that rates of disability 6–12 months after evacuation were high and outcomes worse, overall in those service members with mTBI, comparable to those of civilian cohorts or polytrauma patients with mTBI (MacDonald et al., 2014). MacDonald et al. found no substantial differences in cognition between the evacuated personnel with and without a history of mTBI, however rates of PTSD and depression were higher in the mTBI group (MacDonald et al., 2014).

A substantial number of cross-sectional studies have shown higher (nearly double) rates of PTSD in individuals with mTBI, observed in both military (Hoge et al., 2008; Schneiderman et al., 2008; Luethcke et al., 2011; Vasterling et al., 2012; Rosenfeld et al., 2013) and civilian (Bryant et al., 2010; Mayou et al., 2000) settings. Moreover, these findings have been corroborated using prospective study designs in civilians (Roitman et al., 2013) and in active duty service members (Yurgil et al., 2014). In an 10 day and 8 month follow-up of civilians who presented to the emergency room as a result of motor vehicle accidents, some with mTBI (<30 min loss of consciousness) and some without, Roitman et al., showed that those with head injury and loss of consciousness (LOC) had higher levels of PTSD at follow-up. In the Marine Resiliency Study (MRS), a prospective, longitudinal study, of Marines and Sailors assessed at pre-deployment and again at 3–6 months after a 7-month deployment to Iraq or Afghanistan rates of reported prior TBI were 56.8% at the pre-deployment interview, and rates of deployment-related TBI were 19.8%; of the deployment-related TBIs approximately 87.2% were mild (Baker et al., 2012; Yurgil et al., 2014). As was observed in the civilian study, war-related mTBI significantly increased post-deployment PTSD symptom scores, either doubling or nearly doubling the PTSD rates in combatants who, prior to deployment, had been mentally healthy (Yurgil et al., 2014).

These two prospective studies provide accumulating evidence that mTBI is a robust prognostic indicator of subsequent PTSD development, raising the question as to the underlying cause. Whereas heightened emotional salience of traumatic events that involve blast/concussive injuries versus those without may, in part, provide an explanation for higher PTSD rates after mTBI, another likely, or perhaps even primary explanation may be that mTBI associated structural and functional brain changes increase vulnerability for development of mental disorders such as PTSD (Yurgil et al., 2014). Damage of the mTBI prefrontal cortical networks implicated in PTSD has been suggested as a possible cause of the increased vulnerability (Hoffman and Harrison, 2009; Yurgil et al., 2014).

Pre-clinical studies, as described below, focused on the pathophysiology and mechanisms of neurotrauma may contribute important information regarding mTBI associated brain changes that may contribute to PTSD development. These studies are needed to form a solid scientific basis

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for understanding observed clinical outcomes, and to inform clinical decision making and biomarker development.

2. Preclinical

The use of improvised explosive devices (IEDs) in contemporary asymmetric warfare has changed the scene and spectrum of TBI at the battlefield. The signature TBI has changed from penetrating during the war in Vietnam to blast induced TBI. At the same time, new equipment for body protection has increased the survival rate after TBI at the battlefield. A blast injury is a complex type of physical trauma and includes a variety of injuries, ranging from mild to lethal. The majority of blast induced TBI fall into the category of mild TBI (Hoge et al., 2008) and several groups have tried to develop relevant experimental models for mild blast TBI. It is not possible to make a full review of all experimental research with a focus on blast induced TBI here. However, it is important to underscore the different physics in blast TBI and TBI in a civilian setting, more frequently a result of blunt trauma injury. Extreme forces and their complex propagation characterize blast TBI.

Injury from blast can result from secondary, tertiary and even quaternary effects as well as the primary supersonic pressure wave produced by the blast, all of which have been studied in brain injury models. Secondary effects, due to the impact of flying objects, such as shrapnel fragments, can generate penetrating injuries. The proportion of such injuries was larger in previous conflicts, but seems to have been reduced by improvements in helmet construction. Tertiary effects of blast result from acceleration/deceleration trauma, which may result in tissue shearing and diffuse injuries, such as DAI (diffuse axonal injuries). If the trauma is rotational, the position of the axis of rotation will be an important factor in the injury mechanism and areas at a greater distance from this axis will sustain larger forces. Quaternary effects of blast result from heat, smoke or emission of electromagnetic pulses from detonations (Lee et al., 2011).

If the injury is associated with fragment penetration this will induce more severe focal injury, with subsequent diffuse secondary injuries due to propagation of pressure waves and temporary cavities. Secondary traumatic brain damage occurs as a complication of the different types of TBI and includes ischemic and hypoxic damage, swelling, raised intracranial pressure and infection. The secondary TBI is potentially partly reversible with adequate treatment. Many clinical TBI patients suffer from multiple injuries, i.e. pulmonary lesions or amputations, which can have effects on the outcome of the TBI (due to hypoxia or systemic inflammation). The complexity of the clinical injury and the fact that exposure data seldom are available has created a need for experimental research on biological effects of blast. One early example of this research is the PhD thesis by Carl-Johan Clemedson "*An experimental study on air blast injuries*" (Clemedson, 1949). During the 65 years that have passed since that publication, a considerable number of animal models have been proposed suitable for research on blast induced TBI. The primary blast wave is the propagation of a supersonic pressure wave with short duration. The threshold for injuries is determined by factors such as peak pressure, duration and shape of the wave (reflections, underpressure etc.). Models for better understanding the primary blast wave include (1) open field exposure, (2) blast tubes for explosives and (3) Shock tubes with compressed air or gas.

- *Open field exposure*. Examples here are the large-scale classical experiments in the U.S. in desert areas and ponds, employing large sets of animals of different species and sizes. These experiments determined thresholds for bleeding in air filled organs such as the lungs and intestines, but the potential effects on the central nervous system were not assessed. For simple waveforms, i.e. the Friedländer type of wave, dose response curves (the Bowen curves) were determined.
- *Blast tubes for explosives*. During 1950s large size blast tubes were created to study effects of wave forms relevant to nuclear detonations, i.e. comparatively long duration of the primary peak. The tubes were often used to study how construction details such as doors could withstand a blast wave. One exception was the studies by Clemedson at the Swedish FOA (Swedish Defence Research Establishment) using a smaller blast tube (Clemedson and Criborn, 1955) in which a charge of plastic explosive was used. These types of systems are still in use, but have been outnumbered by shock tube systems.
- *Shock tubes with compressed air or gas*. Systems with compressed air were used already in the 1950s (Celander et al., 1955). Most systems comprise two chambers, separated by a membrane. The gas is loaded into an overpressure chamber (the driver section), which is separated from the main compartment (driven section) by a membrane (diaphragm). The object, i.e. the experimental animal is positioned somewhere in the main section. The operator of the system can rupture the diaphragm at a predetermined pressure and the compressed gas enters the main section as a blast wave. The main section is usually several meters long. If more than one overpressure chamber is positioned in a series rather complex waveforms can be created (Cernak et al., 2011). One advantage with this type of shock tube is the absence of quaternary blast effects and other disadvantages of explosives. However, this advantage can also be regarded as a disadvantage.

One significant problem with the variety of experimental models for blast TBI is that it is very difficult to actually compare the different models. For example, there is no real consensus for monitoring of pressure curves for different models for primary blast. Researchers seem to disagree about recording techniques to access peak pressure, duration and acceleration movements. Blast waves cause damage by a combination of the compression of the air in front of the wave and the subsequent wind, but there is no real consensus on how to represent such parameters in the laboratory situation. Experimental models should be carefully validated in terms of physical parameters (Antona-Makoshi et al., 2014) and outcome (morphologic, functional, molecular and gene expression changes) (Risling et al., 2011). All methods that could facilitate a good translation to clinical data (serological biomarkers, neurophysiolgy and imaging) are recommended.

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Dr. Ibolja Cernak has shown that blast TBI can be a systemic reaction to blast (Cernak, 2010). General inflammatory reactions from the primary blast can contribute to the reactions of the brain. The propagation of pressure waves through the body in blast trauma is still a subject of disagreement. Important data can be retrieved by carefully planned experiments utilizing incomplete body protection (Cernak, 2010). The importance of repeated mild TBI for development of late development of neurodegenerative disease has been documented in sports medicine (Guskiewicz et al., 2005) and repeated injuries will undoubtedly be included in a number of protocols for research on blast TBI. Studies on operators in breaching training can provide a very interesting strategy to collect good exposure data and biomarkers after repeated controlled detonations (Tate et al., 2013). One central problem is that exposure data from actual clinical situations are lacking. Acceleration probes mounted in helmets may help to solve this problem and if the same type of sensors will be implanted for use in animal experiments translation of data may be facilitated.

As noted above, veterans with histories of blast-induced mTBI who have been exposed to explosions are more likely to have headaches, features of migraine, more severe pain, PTSD, and impaired sleep with nightmares. It is difficult to achieve a good representation of such parameters in the evaluation of animal experiments for blast TBI, i.e. blast models that make use of rodents and pigs. Refined behavioral tests with a high sensitivity for stress reactions similar to posttraumatic stress will be important in the future work with blast (Kamnaksh et al., 2011; Kovesdi et al., 2011; Kwon et al., 2011). Additional experiments are required to enable an understanding of the co-morbidity of TBI and PTSD. Such experiments could be combined with biomarker sampling, behavior analysis such as the Forced Swim Test and functional imaging. A recent study has revealed significant changes in catecholamines and serotonin in rodents exposed to a mild TBI (Kawa et al., 2014).

One way to accomplish a better translation between animal experiments and the clinic would be to employ the same methodology for analysis. Imaging, e.g. with MRI (Kamnaksh et al., 2014) or magnetoencephalogram (MEG), and systematic use of biomarkers can be used in both settings and help to bridge the gap between the lab bench and the hospital bed (Agoston et al., 2012). It is important to consider that the limited size of rodent brains creates a demand for good resolution in the imaging technique. Strain differences between different rodents may create difficulties in the interpretation of biomarkers. Different timetables for injury induced changes in biomarkers between rodents and humans should also be considered. Computer based reconstruction of clinical injuries and exposure in the experimental models can help to narrow the knowledge gap between experiments and clinical observations (Kleiven, 2007). Fine-tuning of the finite element models would need to include both tissue properties and a proper representation of fiber tracts. Modeling however, has limited use if the predictions cannot be validated by actual biological observations. The different geometrical shape of the rodent brain and humans can create obstacles in modeling. It is therefore advantageous if data from larger animals, such as pigs, are available also.

The Vietnam Head Injury study can also be used as an example of translation. Outcome data from a large cohort of patients that survived penetrating brain injuries has been analyzed during more than 35 years (Raymont et al., 2011). This is probably one of the most detailed follow-up neurotrauma studies that has ever been conducted. This material has been employed to reveal the importance of the growth factor BDNF on the outcome of the injury (Rostami et al., 2011) an observation that later was brought back for further investigation in a suitable animal model (Rostami et al., 2014).

In summary, blast TBI involves complex energy transfer and several possible mechanisms. The primary blast wave, acceleration generated tissue strain, smoke and heat can induce both mild TBI and more severe injuries. It is essential to have a good control on physics during animal experiments on blast induced TBI. Translation to clinical situations can be facilitated if serological biomarkers and advanced imaging techniques can be used.

3. Clinical

In humans, the observation that repeated mild injuries could result in chronic traumatic encephalopathy (CTE) (Lakis et al., 2013; Stein et al., 2014). has influenced scientists to start experimental projects on repeated mild blast induced TBI (Petraglia et al., 2014a,b; Glushakova et al., 2014; Goldstein et al., 2014).

Also there is now evidence that changes in endocrine functions and regulation can show significant changes in TBI patients. Serum levels of melatonin can probably contribute to long-term sleep disturbances in TBI patients (Seifman et al., 2014). It has been observed that pituitary dysfunction may be one of the consequences of blast TBI (Baxter et al., 2013) and this observation should be analyzed also in experimental models for blast.

Of course, there is an extensive pre-clinical and clinical literature showing abnormalities in neuroendocrine function in PTSD, review of which is beyond the focus of this paper (de Kloet et al., 2006; Krystal and Neumeister, 2009). Plasma melatonin levels collected 48 h after a trauma in Australian troops are reported to predict later PTSD, but, a recent study that assessed polysomnography with simultaneous blood sampling in returning Dutch troops observed no PTSD-related plasma melatonin abnormalities, despite sleep disturbance (McFarlane et al., 2010; van Liempt et al., 2013). The same Dutch study, though, showed evidence for a link between hypothalamic–pituitary–adrenal (HPA) axis abnormalities that were observed and sleep disturbance in the troops with PTSD (van Liempt et al., 2013).

To date most neuroendocrine research has focused on either PTSD or mTBI and has not grappled with the complexities of disentangling effects of possible co-occurring disorders. Clearly, going forward, as our ability to detect post-head injury residual brain injury improves, it will be important to carefully characterize diagnostic status (mTBI, PTSD and the combination) in endocrine and autonomic research in military cohorts in order to fully tease apart biomarkers related to separate (mTBI, PTSD) and combined (mTBI and PTSD) status, given the high co-occurrence of both disorders in deployed troops.

Inflammatory reactions and cell death after TBI can be different in males and females (Gunther et al., 2015).

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Such differences can possibly be related to endocrine functions. Also metabolic functions can be altered after TBI. For example, changes in the cytochrome p450 superfamily of metabolic enzymes could influence the metabolism of inflammatory mediators, drugs and hormones (Birnie et al., 2013). Metabolic abnormalities have been proposed, but are yet to be studied in humans with PTSD (Naviaux, 2014).

At present, it is unclear why similar mTBI events can lead to dramatic neurobehavioral decompensation with persistent PCS in some individuals, but not in others (Jeter et al., 2013). Retrospective and prospective studies of combat-related TBI show that most (<85%) deployment-related TBIs are mild (MacGregor et al., 2011; Yurgil et al., 2014). But diagnoses are based primarily on the characteristics of the acute clinical sequelae following the injury. The subtle, scattered and varied lesion(s) usually go undetected by conventional CT, and they are often unobservable on conventional MRI as well (Van Boven et al., 2009; Bigler and Orrison, 2004; Johnston et al., 2001; Kirkwood et al., 2006). Teasdale and Jennet showed that intracranial lesions in mTBI are detected by conventional neuroimaging techniques in only 4%, 16%, and 28% of patients with Glasgow Coma Scale scores (GCS) (Teasdale and Jennett, 1974) of 15, 14, and 13, respectively (Culotta et al., 1996).

Usually, the persistent PCS and cognitive deficits in TBI patients cannot be explained solely by focal pathology. DAI is a major contributor to these deficits and is commonly induced by sudden acceleration-deceleration or rotational forces. In a rodent TBI model, axonal injury was the most prominent feature following blast exposure (Garman et al., 2011). In humans, the subsequent tissue injury is characterized by axonal stretching, inflammation, disruption, and separation of nerve fibers, although axotomy is relatively rare in even severe TBI (Adams et al., 1989; Basser and Pierpaoli, 1996; Gennarelli et al., 1982; Xu et al., 2007). Conventional CT and MRI are primarily sensitive to blood from nearby torn capillaries, rather than axonal damage itself, hence they underestimate the presence of DAI, especially in mTBI. New approaches using diffusion tensor imaging (DTI), positron emission topography (PET), and macromelecular proton fraction (MPF) have showed promising capability in detecting injuries and/or abnormalities that are not visible in CT and MRI (e.g., Petrie et al., 2014; MacDonald et al., 2011; Davenport et al., 2012; Shenton et al., 2012).

Magnetoencephalogram (MEG) is a non-invasive functional imaging technique that directly measures the magnetic signal due to neuronal activation in gray matter (GM) with high temporal resolution (<1 ms) and spatial localization accuracy (2–3 mm at cortical level) (Leahy et al., 1998). MEG demonstrates sensitivity to abnormal neuronal signals resulting from axonal injuries. Neurophysiological studies in animals have established a solid connection between pathological delta-wave (1–4 Hz) generation in GM and axonal injuries in WM. Gloor et al. showed that polymorphic delta-band slow-waves produced by (white matter) WM axonal lesions in the cat were localized to the GM area of cortex overlying the lesion (Ball et al., 1977; Gloor et al., 1977). They also found that pathological delta-waves can be induced by the administration of atropine in the WM (Schaul et al., 1978). It is known that atropine is a competitive antagonist of acetylcholine receptors and can block and/or limit the cholinergic pathway. These experiments concluded that cortical de-afferentation was an important factor in abnormal delta-wave production, owing to WM lesions (i.e., axonal injury) and/or defects in the cholinergic pathway (Schaul, 1998). In the human brain, the projections of cholinergic pathways highly overlap with the WM fiber tracts (Selden et al., 1998), which make the cholinergic pathways similarly susceptible as the WM tracts to TBI.

Human studies by Lewine et al., and our laboratory showed that the brains of mTBI patients generate abnormal low-frequency magnetic fields that can be measured and localized by resting-state MEG (Huang et al., 2009, 2012; Lewine et al., 1999, 2007). MEG was also found to be more sensitive than conventional MRI or EEG in detecting abnormalities in mTBI patients (Lewine et al., 1999, 2007). Unlike normal resting-state MEG data, which is dominated by neuronal activity with frequencies above 8 Hz, injured neuronal tissues (due to head trauma, brain tumors, stroke, and epilepsy) generate abnormal focal or multi-focal low-frequency neuronal magnetic signals (delta-band 1–4 Hz, or theta-band 5–7 Hz) that can be directly measured and localized using MEG (Baayen et al., 2003; de Jongh et al., 2003; Decker and Knott, 1972; Lewine et al., 1999; Lewine and Orrison, 1995; Nagata et al., 1985; Vieth et al., 1996). While TBI is not the only neurological disorder that generates abnormal slow-wave, in practice, brain tumors, stroke, and epilepsy can be easily ruled out based on structural imaging (i.e., CT and MRI for tumor and stroke) and medical history (for epilepsy).

Fig. 1 shows an example of the abnormal resting-state MEG slow-wave findings in one of studies (Huang et al., 2009) from a chronic mTBI patient (sport injury) with persistent PCS including: pressure headaches, dizziness, fatigue, memory problems, difficulty falling asleep, and changes in speech and language. Multiple clinical CT and MRI scans were all negative. Fig. 1 shows that the abnormal MEG slow-waves came from: (1) lateral superior-posterior left temporal lobe, and (2) an area containing three sub-regions in the ventral right temporal and occipital lobes (Huang et al., 2009). Deafferentation due to axonal injuries was most likely the cause of slow-waves in these GM regions which was confirmed by Diffusion Tensor Imaging (DTI) tractography analysis (Huang et al., 2009).

In a separate region-of-interest (ROI) study from our lab (Huang et al., 2012), we assessed abnormal resting-state MEG slow-wave (1–4 Hz) generation from 96 cortical regions from three TBI groups: 23 mild TBI patients exposed to combat-related blasts, 22 mild TBI patients with non-blast causes (sports, motor vehicle accidents, fall, and assault), and 10 moderate TBI patients with non-blast causes. The normative database for the ROI-based MEG slow-wave power was established using data from 44 healthy control subjects. Fig. 2 shows a conservative threshold (horizontal line) in which all healthy control subjects' slow-wave measures were below this level (0% false-positive rate). With such a threshold, the positive detection rates were 96% for mild blast-induced TBI patients (22 out of 23), 77% for the mild non-blast TBI patients (17 out of 22), and 100% for the moderate TBI patients (10 out of 10). When we combined the blast-induced and non-blast mild TBI groups together, the correct diagnostic rate was about 87% for the combined mild TBI group. This study provides a foundation for using

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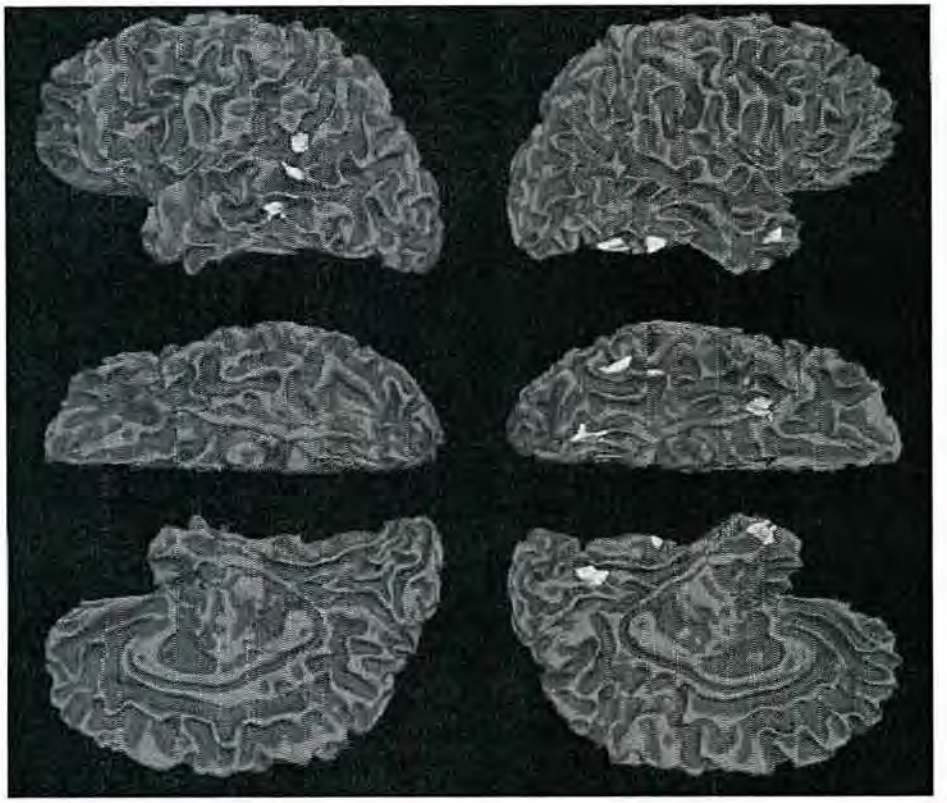

Figure 1 Abnormal MEG slow-waves. (1) Left column: L lateral superior-posterior temporal region. (2) Right column: R inferior-temporal areas. Three rows are lateral-, ventral-, and middle-views, respectively.

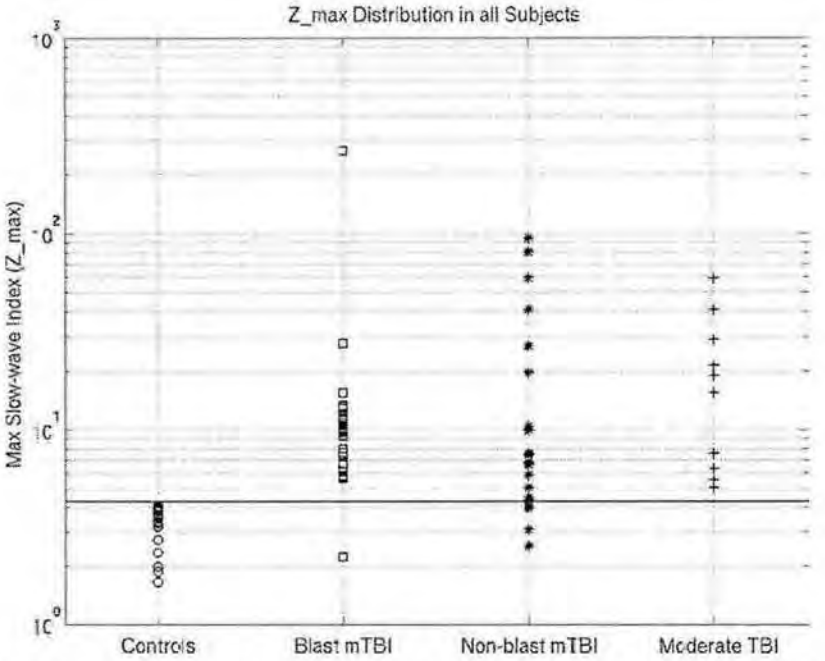

Figure 2 Frequency-domain MEG low-frequency source imaging power are plotted separately for (1) healthy control, (2) mild blast-induced TBI, (3) mild non-blast-induced TBI, and 4) moderate TBI groups. The y-axis is in logarithm scale.

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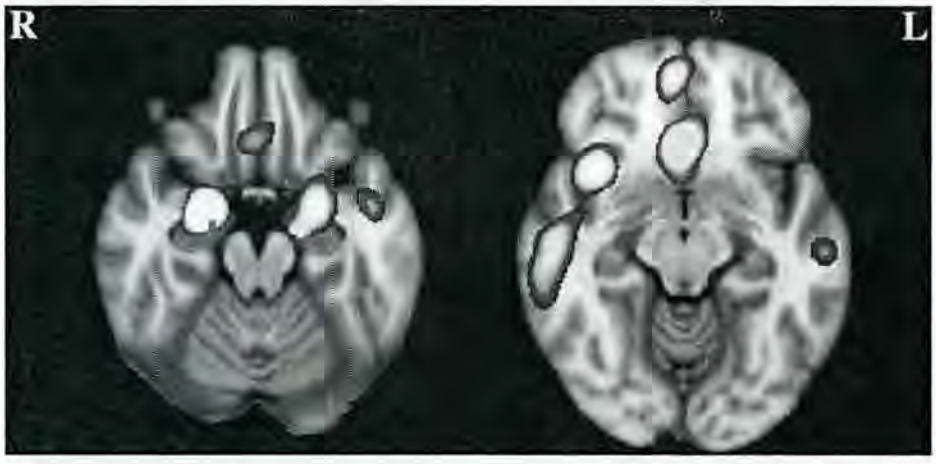

Figure 3 MEG source imaging in beta and gamma bands shows hyperactivities (PTSD > Ctrl, red-hot color) from amygdala/anterior hippocampus and hypoactivities (PTSD < Ctrl, blue-cold color) from the vmPFC, with corrected $p < 0.05$ in beta-gamma band.

MEG low-frequency source imaging as potential biomarker to assist in the clinical diagnosis of mild TBI.

Recently, we expanded the above ROI-based approach by developing a voxel-based whole-brain MEG slow-wave imaging approach, Fast-VESTAL (Huang et al., 2014a), for detecting abnormality in patients with mTBI on a single-subject basis (Huang et al., 2014b). A normative database of resting-state MEG source magnitude images (1–4 Hz) from 79 healthy control subjects (68 civilians and 11 active-duty military service members) was established for all brain voxels. The high-resolution MEG source magnitude images were obtained by our recent Fast-VESTAL method. In 84 mTBI patients with persistent post-concussive symptoms (36 from blasts, and 48 from non-blast causes), our method detected abnormalities at the positive detection rates of 84.5%, 86.1%, and 83.3% for the combined (blast-induced plus with non-blast causes), blast, and non-blast mTBI groups, respectively. We found that prefrontal, posterior parietal, inferior temporal, hippocampus, and cerebella areas were particularly vulnerable to head trauma. The results also showed that MEG slow-wave generation in prefrontal areas positively correlated with personality change, trouble concentrating, affective lability, and depression symptoms.

Amygdala, vmPFC, and hippocampal regions implicated in pre-clinical fear processing are thought to be dysfunctional in PTSD (Rauch et al., 1998, 2006). Functional neuroimaging findings using positron emission topography (PET) and functional magnetic resonance imaging (fMRI) suggest that individuals with PTSD exhibit hyperresponsive amygdala activity to trauma or fear-related stimuli (for review, see Shin and Liberzon, 2010), during emotionally neutral tasks (Bryant et al., 2005; Shin et al., 2004b), and even at rest (Chung et al., 2006; Semple et al., 2000). A hyperresponsive amygdala contributes to the exaggerated fear response characteristic of PTSD (Anderson et al., 2003). Conversely, PTSD has been associated repeatedly with hyporesponsive vmPFC (for review, see Hughes and Shin, 2011). Hyporesponsive PFC, as well as reduced connectivity to the amygdala (Jin et al., 2013; Shin et al., 2004a) may indicate insufficient inhibitory control over exaggerated fear responses. Lastly, abnormal hippocampal function (Corcoran and Maren, 2001) and reduced connectivity to the amygdala (Dolcos et al., 2004; McGaugh, 2004) may underlie impairments in contextual memory processing and the ability to inhibit intrusive memories (Shin et al., 2004a), although findings have been mixed (Hughes and Shin, 2011). A recent resting-state fMRI study showed increased activity in amygdala and reduced spontaneous neural activity in the dorso-lateral PFC (DLPFC) (Yan et al., 2013). However, the authors found no evidence of abnormal resting-state fMRI signal in the vmPFC.

Recently, we used resting-state MEG to study 16 OEF/OIF active-duty military and veteran participants with PTSD, and 23 age-matched healthy control subjects. Among the control subjects, 12 were active-duty military personnel deployed to Iraq and/or Afghanistan and 11 were civilians without military training. Voxel-based whole brain MEG source magnitude images were obtained using our new Fast-VESTAL high-resolution MEG source imaging method (Huang et al., 2014a). Fig. 3 shows that for beta-gamma band (15–80 Hz), increased MEG activity in PTSD (hyperactivities, PTSD > controls) was generated from bilateral amygdala/anterior hippocampus, whereas decreased MEG activity was generated from the vmPFC. The MEG results were consistent with the theory that reduced inhibition (hypoactivity) from the vmPFC and hyperactivity in the "fear network" including the amygdala/anterior hippocampus are closely related. These data suggest that MEG imaging may accurately visualize brain evidence of PTSD, but more research is needed to fully develop MEG as a potential biomarker for PTSD.

As discussed earlier, TBI significantly potentiates PTSD development. Here we present some preliminary data of using a voxel-based MEG source imaging approach to evaluate the potentiation of PTSD by an mTBI. The new aspect of the study is to provide much needed information regarding exactly what brain regions that are part of the abnormal PTSD neurocircuitry are also particularly vulnerable to mTBI. Such information may contribute to more effective treatments for veterans with comorbid mTBI and PTSD, and guide the development of preventive strategies in PTSD.

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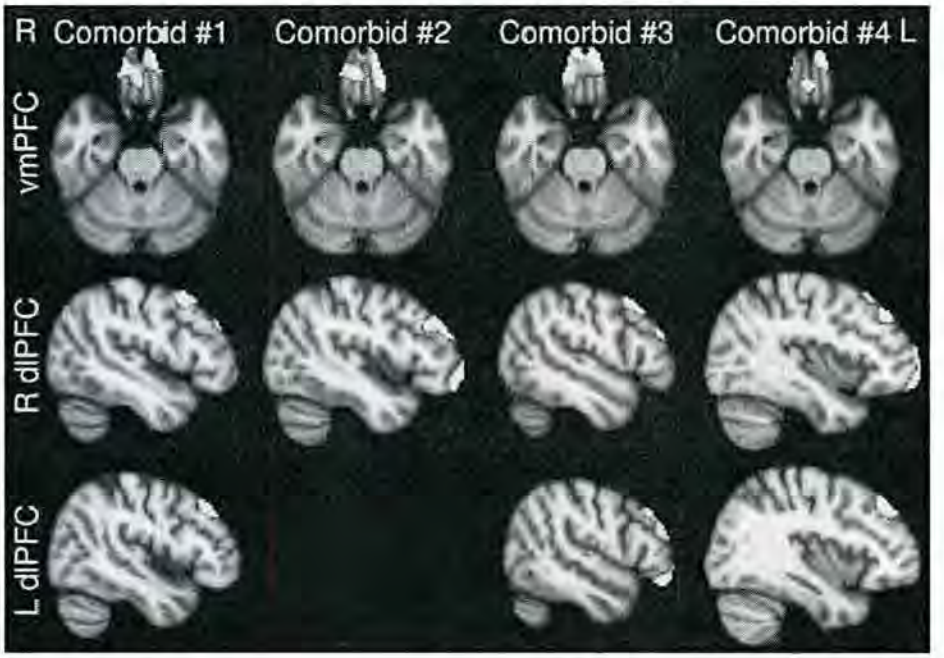

Figure 4 MEG source imaging showing slow-wave generation in four patients with comorbid mTBI and PTSD from vmPFC and dlPFC suggests that mTBI may potentiate the development of PTSD.

Fig. 4 shows preliminary data of abnormal MEG slow-waves from 4 patients with comorbid mTBI and PTSD. All patients showed abnormal slow-waves (1–4 Hz) from vmPFC, 3 out of 4 showed bilateral dlPFC abnormal slow-waves, and 1 showed right dlPFC abnormal slow-wave generation, indicating potential injuries due to mTBI. The slow-wave generation suggests mTBI in these PFC areas. In addition, similar to the preliminary result from the group of 25 PTSD subjects, these 4 comorbid patients also showed MEG hypoactivity from vmPFC and dlPFC in high frequency bands when compared with the HCs, similar to the findings presented in Fig. 3. In this comorbid group, the co-existence of abnormal MEG slow-wave (mTBI component) and hypoactivity from vmPFC and dlPFC in high frequency bands (PTSD component) suggests the mTBI injuries in PFC may result in a lack of inhibition from PFC to other areas of the PTSD neurocircuitry. This preliminary data, thus, provides evidence of abnormal slow-wave generation in these PFC areas due to mTBI and the potentiation of PTSD.

4. Limitations of this study

There are several limitations of this study: One concern with neuroimaging studies, including many recent studies involving OIF/OEF veterans, are the reliance on convenience samples and the use of control groups that do not always provide adequate scientific comparisons (Hoge and Castro, 2011, 2014). Next, we have focused on the potentiation of PTSD development due to mTBI. Deployment to Iraq or Afghanistan, as in past wars, is certainly associated with non-specific generalized physical and cognitive health effects, and it is likely that there are multiple causes for these health concerns beyond mTBI and PTSD (e.g. prolonged periods of sleep deprivation, combat intensity, intense physical strain on the body from harsh foot patrols, depression, repetitive load-bearing injuries, etc.) (Hoge and Castro et al., 2014). Also, deployment experiences are highly variable. Roughly two-thirds of OIF/OEF service members worked principally inside heavily fortified compounds with limited exposure to war-zone stressors (other than random indirect fire). The other one-third, mostly infantry brigades, have done the lion's share of the direct combat heavy lifting, but even in those units there is high variability of exposure experiences. Clearly service members in units that experience more direct combat, higher levels of sleep deprivation, and more non-head injuries will likely have higher rates of non-specific abnormalities on functional neuroimaging (to include vmPFC or dlPFC) than comparison groups that did not have this level of deployment intensity, and thus it is important in future studies to consider these factors when designing studies that attempt to look at the neuroimaging associated with blast, mTBI or PTSD cases. In the present study, our control group for the MEG slow-wave source imaging study contains a mix of active-duty military and civilian subjects. This mix was necessary since we assessed both subjects with blast-induced mTBI as well as civilian mTBI subjects without blast exposure. Nevertheless, the imbalance of active-duty military subjects between the control and blast mTBI groups remains a limitation.

Also, as noted previously, the focus of this paper is on mTBI and on MEG imaging, thus an exploration of the full range of biomarkers, in particular the large literature on blood-based biomarkers in PTSD, was beyond the scope of this paper. The common co-existence of mTBI and PTSD pose further significant challenges for blood-based biomarker research in both mTBI and PTSD in military and veteran populations, since co-occurrence of either must be assessed,

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and considered during study enrollment, and is a limitation of current research. Since blast exposure is a risk for PTSD development, and there is significant overlap in PTSD and mTBI, an ultimate goal of the MEG research will be the development imaging biomarkers for PTSD, and for mTBI on a single subject basis, i.e. diagnostic biomarkers.

5. Conclusion

In summary, TBI is a highly prevalent condition, although the majority of cases of TBI are mild. While the social and fiscal impact of moderate and severe TBI has been well known, until recently, the potential negative impact of mTBI on health has been underappreciated. In contrast to tools available in the 20th century, we now have great advancements in technology that, combined with pre-clinical insights, can support development of improved approaches for clinical visualization of the mTBI injuries, which may provide a basis for a new schema for mTBI diagnosis. With more precise diagnostic approaches, such as high resolution imaging, and cerebrospinal fluid or blood testing, we may ultimately be able to develop imaging and bodily fluid biomarkers for use in prognosis, diagnosis, or as treatment outcome measures.

Conflict of interest

All authors report no further biomedical financial interests or potential conflicts of interest.

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ORIGINAL ARTICLE

Gene networks specific for innate immunity define post-traumatic stress disorder

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The molecular factors involved in the development of Post-Traumatic Stress Disorder (PTSD) remain poorly understood. Previous transcriptomic studies investigating the mechanisms of PTSD apply targeted approaches to identify individual genes under a cross-sectional framework lack a holistic view of the behaviours and properties of these genes at the system-level. Here we sought to apply an unsupervised gene-network based approach to a prospective experimental design using whole-transcriptome RNA-Seq gene expression from peripheral blood leukocytes of U.S. Marines ($N=188$), obtained both pre- and post-deployment to conflict zones. We identified discrete groups of co-regulated genes (i.e., co-expression modules) and tested them for association to PTSD. We identified one module at both pre- and post-deployment containing putative causal signatures for PTSD development displaying an over-expression of genes enriched for functions of innate-immune response and interferon signalling (Type-I and Type-II). Importantly, these results were replicated in a second non-overlapping independent dataset of U.S. Marines ($N=96$), further outlining the role of innate immune and interferon signalling genes within co-expression modules to explain at least part of the causal pathophysiology for PTSD development. A second module, consequential of trauma exposure, contained PTSD resiliency signatures and an over-expression of genes involved in hemostasis and wound responsiveness suggesting that chronic levels of stress impair proper wound healing during/after exposure to the battlefield while highlighting the role of the hemostatic system as a clinical indicator of chronic-based stress. These findings provide novel insights for early preventative measures and advanced PTSD detection, which may lead to interventions that delay or perhaps abrogate the development of PTSD.

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INTRODUCTION

The study of the molecular factors that determine risk and subsequent development of Post-traumatic stress disorder (PTSD) are at the forefront of molecular psychiatric research. A significant number of men and women exposed to severe emotional trauma and loss emerge from these events with persistent PTSD symptoms, such as intrusive imagery, avoidance and hyperarousal, as well as other long-term physical health problems. PTSD affects 7–8% of the general United States (US) population, and is higher among troops recently returned from the wars in Iraq and Afghanistan, with estimates of prevalence as high as 20%.[1] Annual health care costs associated with PTSD in the US have been estimated to be 180 million dollars.[2] Heterogeneity in susceptibility to PTSD suggests that differences at the molecular level (i.e. gene-expression level) may influence an individual's physiological and psychological response to trauma and thus the development of PTSD. A clear understanding of the molecular mechanisms underlying this aberrant response to trauma is required to reduce the substantial morbidity and mortality associated with this disorder.

A number of studies have analyzed blood gene expression and glucocorticoid activity to build more effective models for identifying molecular factors associated to PTSD.[3–12] These studies were recently reviewed by Heinzlemann and Gill,[2] who summarized that the increased expression of inflammatory genes and decreased expression of the genes that regulate inflammation contribute to the onset of PTSD. Specifically, when considering the overlap in results from transcriptomic studies, the decreased expression of *FKBP5* and *STAT5B*, which regulate inflammation, is evident.[4,6,7,9] The majority of these reviewed studies[3–8,11,12] centered transcriptomic analyses on subjects already diagnosed with PTSD, and thus lacked a prospective study design, as well as independent datasets for validation purposes. These studies employ gene expression analysis on pre-determined targets, focusing analyses on the individual gene-level and the putative clinical utilities of the resulting gene-list, without studying the connectivity of these genes at the system-level.

Recent gene-expression network analyses, such as weighted gene co-expression network analysis (WGCNA), aim to integrate expression data across thousands of genes into a higher-order

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system-level context to identify groups of genes within a network whose expressions are highly correlated (i.e. co-expression modules).[13] In doing so, WGCNA provides a powerful unsupervised approach to tackle the molecular complexity that occurs in neurodevelopmental and psychophysiological disorders,[14–19] although has never before been applied to PTSD.

We applied WGCNA to RNA-Seq and microarray peripheral blood leukocyte (PBL) gene expression taken from two independent groups of U.S. Marines, both pre- and post-deployment to conflict zones. The primary goal of this analysis was to best characterise the prognostic and diagnostic molecular signatures defining both 'PTSD risk' and 'PTSD' states, while demonstrating the robustness and reproducibility of WGCNA findings across datasets. Instead of identifying differentially expressed genes on a gene-by-gene basis, we constructed unsupervised gene co-expression networks from a combination of case and control data and identified gene co-expression modules within these networks. Modules were first assessed for containing differentially expressed genes, tested for their association with PTSD, and finally subjected to functional enrichment analysis. In this manner, we then assessed whether the PTSD-associated modules were detected in our second non-overlapping dataset of U.S. Marines to demonstrate a significant and consistent association of our findings. We conclude that prospectively profiling the transcriptome of U.S. Marines pre- and post-deployment to conflict zones, using a co-expression analysis approach is a promising strategy for identifying and studying the functions of causal and consequential molecular factors in PTSD development, with particular value in reproducing results across independent datasets of U.S. Marines.

SUBJECTS AND METHODS

Sample collection and datasets

All subjects were male and participants in either the Marine Resilience Study (MRS) or the Marine Resiliency Study II (MRS II), prospective studies of well-characterized U.S. Marines scheduled for combat deployment to Iraq or Afghanistan, with longitudinal follow-up to track the effect of combat stress.

Dataset 1—Whole blood was obtained from 124 MRS II U.S. Marine participants who served a seven month deployment. Blood was drawn 1-month prior to deployment and again at 3-months post-deployment for each participant. Each blood sample (10 ml) was collected into an EDTA-coated collection tube, RNA was isolated from peripheral blood leukocytes using LeukoLOCK Total RNA isolation and sequenced using the Illumina Hi-Seq 2000.

Dataset 2—For validation, data were compared to an independently generated gene expression data-set from a separate, non-overlapping, group of 50 MRS U.S. Marine participants (Glatt *et al.* 2013, previously published pre-deployment data[12]). Blood samples were treated in an identical fashion as described above, however final RNA was hybridized to the Affymetrix Hu-Gene 1.0 ST Array.

PTSD diagnosis

At the time of each blood draw, PTSD symptoms were assessed using a structured diagnostic interview, the Clinician Administered PTSD Scale (CAPS).[20–23] Using the criteria from the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (2000),[24] diagnosis for partial or full PTSD was defined as a threat to life, injury, or physical integrity (Criterion A1) and the presence of at least one re-experiencing symptom and either three avoidance symptoms or two hyperarousal symptoms, or two avoidance symptoms plus two hyperarousal symptom.[25–27] Symptoms must have occurred at least once within the past month (frequency $\geqslant 1$) and caused a moderate amount of distress (intensity $\geqslant 2$).

Subject selection

A subset of MRS II study participants were pre-selected for RNA-Seq analysis. First, at pre-deployment, all participants had to be symptom free, with no PTSD diagnosis and a CAPS $\leqslant 25$. Second, at post-deployment, participants who fulfilled criteria for partial or full PTSD diagnosis were designated the PTSD group. Third, participants with post-deployment CAPS $\leqslant 25$ that matched the post-deployment PTSD group on variables of combat exposure, age and ethnicity were designated the 'control' group. Under these criteria, all paired subjects were stratified into two groups based upon CAPS scores at 3-months post-deployment (Table 1, Supplementary Table 1). If a U.S. Marine participant developed PTSD following trauma-exposure at 3-months post-deployment, their pre-deployment sample would be included in the 'PTSD-risk' group. Likewise,

Table 1. Recorded clinical parameters from U.S. Marines assessed at pre- and post-deployment for Dataset 1

Time point	*Pre-Deployment*			*Post-Deployment*		
	PTSD Cases (N = 47)	*Controls (N = 47)*	*P-value*	*PTSD Cases (N = 47)*	*Controls (N = 47)*	*P-value*
Age	22.15 ± 2.53	22.42 ± 3.92	0.682	23.14 ± 2.52	23.42 ± 3.92	0.685
Alcohol	2.08 ± 1.55	1.62 ± 1.33	0.124	1.79 ± 1.32	1.54 ± 1.11	0.318
Tobacco	1.75 ± 1.62	0.97 ± 1.51	0.02	1.69 ± 1.69	1.02 ± 1.47	0.042
WC adj.	1.65 ± 0.13	1.72 ± 0.13	0.015	1.68 ± 0.14	1.75 ± 0.12	0.012
PCL	21.29 ± 4.72	18.33 ± 2.27	0.0001	42.38 ± 11.09	20.94 ± 3.87	5.37E-22
CAPS total	11.39 ± 7.23	6.75 ± 6.90	0.002	53.17 ± 15.08	10.04 ± 7.26	5.99E-32
CAPSBs	1.00 ± 1.91	0.54 ± 1.92	0.245	14.9 ± 7.25	1.54 ± 2.37	6.29E-21
CAPSCAs	0.54 ± 1.11	0.10 ± 0.51	0.015	5.31 ± 4.57	0.85 ± 2.08	1.88E-08
CAPSCN1s	1.10 ± 2.23	0.97 ± 2.88	0.813	9.17 ± 5.32	1.19 ± 2.87	1.21E-14
CAPSDs	8.39 ± 5.66	4.58 ± 4.98	0.001	22.6 ± 6.7	6.42 ± 4.79	5.97E-24
CAPSCs	2.00 ± 2.73	1.62 ± 3.66	0.571	15.67 ± 7.23	2.08 ± 3.66	7.15E-20
Prior Deployment	19	16	0.6699	—	—	—
TBI	—	—	—	30	21	0.097
CES PBE mean	—	—	—	0.63 ± 0.25	0.53 ± 0.12	0.02
Caucasian	26	26	1	—	—	—
African American	4	4	1	—	—	—
Native American Mexican	13	15	0.822	—	—	—
Asian & Other	5	3	0.714	—	—	—

Abbreviations: Alcohol, alcohol consumption; CAPS total, CAPS total score; CAPSBs, re-experiencing subscale; CAPSCAs, symptoms of avoidance; CAPSCN1s, symptoms of numbing; CAPSCs, subtotal C subscale; CAPSDs, hyper-arousal subscale; CES, combat exposure scale; PBE, post battle experience; PCL, PTSD symptom check list; TBI, traumatic brain injury; Tobacco, tobacco use; WC adj., waist circumference was adjusted for height; -, not applicable. Significance was assessed with a Student's two-tailed *t* test for continuous variables and fishers exact test of proportions for binary variables. (Average ± standard deviation).

if a subject avoided PTSD symptoms at 3 months post-deployment their sample at pre-deployment was included in the 'control' group.

Data pre-processing

All data were pre-processed by normalization, filtering genes with low expression values, and removing any outliers which may bias down-stream analysis. Final subject numbers resulted in 94-paired subjects (47 paired cases and 47 paired controls) in *Dataset 1* and 48 paired subjects (24 paired cases and 24 paired controls) in *Dataset 2*. To compare findings from RNA-Seq data in *Dataset 1* to microarray data in *Dataset 2*, genes found only on both platforms ($N = 10\,184$) passed into our subsequent analysis (see Supplementary File for more detailed information).

Differential gene expression analyses

Differentially expressed genes were assessed using the moderated *t*-test in edgeR[28] and LIMMA[29] packages for RNA-Seq and microarray data, respectively, and unless otherwise specified, the significance threshold was a nominal *P*-value < 0.05. A nominally significant *P*-value was used to yield a reasonable number of genes to include within network analyses. Differential expression analyses were performed on 10 184 genes between pre-deployment PTSD case and control groups, and again between post-deployment PTSD case and control groups (see Supplementary File for more detailed information).

Gene network construction and module detection

Signed co-expression networks were built using weighted gene co-expression network analysis (WGCNA)[13] in R. A total of 10 184 genes were used to construct each network. To construct the networks, the absolute values of Pearson correlation coefficients were calculated for all possible gene pairs and resulting values were transformed so that the final matrix followed an approximate scale-free topology (see Supplementary File for detailed information). The WGCNA dynamic tree-cut algorithm was used to detect network modules. In order to determine which modules, and corresponding processes were most associated to PTSD related states, we ran singular value decomposition on each module's expression matrix and used the resulting module eigengene (ME), which is equivalent to the first principal component,[13] to represent the overall expression profiles for each module. For each gene in a module, module membership (*kME*) was defined as the correlation between gene expression values and *ME* expression. Genes with high *kME* inside co-expression modules are labeled as hub genes.[13] *GS* was calculated as the $-\log_{10}$ of the *P*-value generated for each gene within a particular module using a moderated *t* test and is a measure of the strength of differential gene expression between PTSD cases and controls. *MS* was calculated as the average *GS* within each module (see Supplementary File for more information).

Statistical analyses

All gene-set overlap analyses were performed by assessing the cumulative hypergeometric probability using the *phyper* function in R.

Enrichment analyses

Module enrichment was assessed three ways. First, general module enrichment categories were obtained using gene ontology biological processes from the DAVID database[30] (http://david.abcc.ncifcrf.gov/). Second, specific module enrichment categories were obtained using the WGCNA function userlistEnrichment[31] using modules as input-lists and curated Reactome NCBI Biosystems pathways and terms[32] as user-defined lists. Finally, we downloaded the highly expressed, cell specific (HECS) gene expression database compiled by Shoemaker *et al.*[33] to assess cell-type specific enrichment results, here cell-type marker lists were used as a user-defined lists. All module genes were used for enrichment analyses using a FDR corrected *P*-value < 0.05 as significant.

Data availability

RNA-Seq and microarray gene expression data are freely available at the Gene Expression Omnibus under the SuperSeries accession number GSE64814 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64814).

Full Methods and any associated references are available in Supplementary Methods.

RESULTS

We analyzed two different gene expression datasets generated from RNA-Seq (*Dataset 1*, Table 1) and microarray (*Dataset 2*, Supplementary Table 1) using peripheral blood leukocyte (PBL) samples taken from U.S. Marines pre- and post-deployment. Following a set of differential gene expression analyses (Supplementary Figure 1), we aimed to characterise the prognostic and diagnostic molecular signatures of PTSD by studying transcriptional differences at the systems-level at pre-deployment and post-deployment separately. Initially, WGCNA was used in *Dataset 1* to assess module preservation between PTSD cases ($N = 47$) and controls ($N = 47$) for the pre- and then the post-deployment time point (see Supplementary File for complete description). This analysis identifies large differences in gene co-regulatory patterns, as being disrupted or created in PTSD cases relative to controls, or vis-versa. However, we observed strong preservation statistics between the two groups indicating similar fundamental gene co-regulation within PTSD cases and controls, suggesting that major changes in the underlying gene-gene connectivity are not a basis for the pathology of this disorder (Supplementary Table 2). As a result we used the higher confidence and completeness of a combined network of case and control data.

Differential module expression post-deployment in Dataset 1

We constructed a gene co-expression network from a combination of PTSD cases ($N = 47$) and controls ($N = 47$) post-deployment using RNA-Seq expression data from *Dataset 1* (Figure 1). This analysis identified nine modules (fully characterised in Supplementary Table 3) that were first examined for enrichment of differentially expressed genes. Two modules (M1A and M1B) were enriched for genes identified as differentially expressed between PTSD cases and controls, reflected by an elevated module significance (*MS*) value (Figure 2a). To determine if the overall expression of modules M1A and M1B were significantly associated with PTSD group status, we calculated differences in module expression using module eigengene (*ME*) values *(See Materials and Methods for complete description of ME)*. Consistent with results using MS, expression of module M1B was significantly higher in the PTSD resilient control group ($P = 0.004$ and Figure 2b) suggesting a positive correlation to PTSD resiliency, meanwhile expression of module M1A was significantly higher in the PTSD

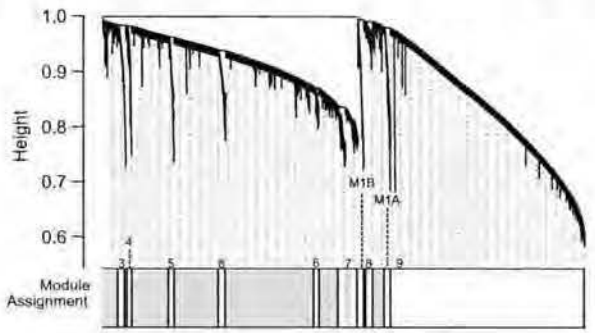


Figure 1. Hierarchical cluster tree and post-deployment module structure in *Dataset 1*. Hierarchical cluster tree (dendrogram) of the combine post-deployment network of PTSD cases ($N = 47$) and controls ($N = 47$) comprising 10 184 genes. Each line represents a gene (leaf) and each low-hanging cluster represents a group of co-expressed genes with similar network connections (branch) on the tree. The first band underneath the tree indicates the nine detected, and subsequently analyzed, network modules. Genes shaded in grey were not assigned to a particular module and represent background noise. For a comprehensive functional annotation of each module and calculation of all significant module-trait relationships see Supplementary Table 3.

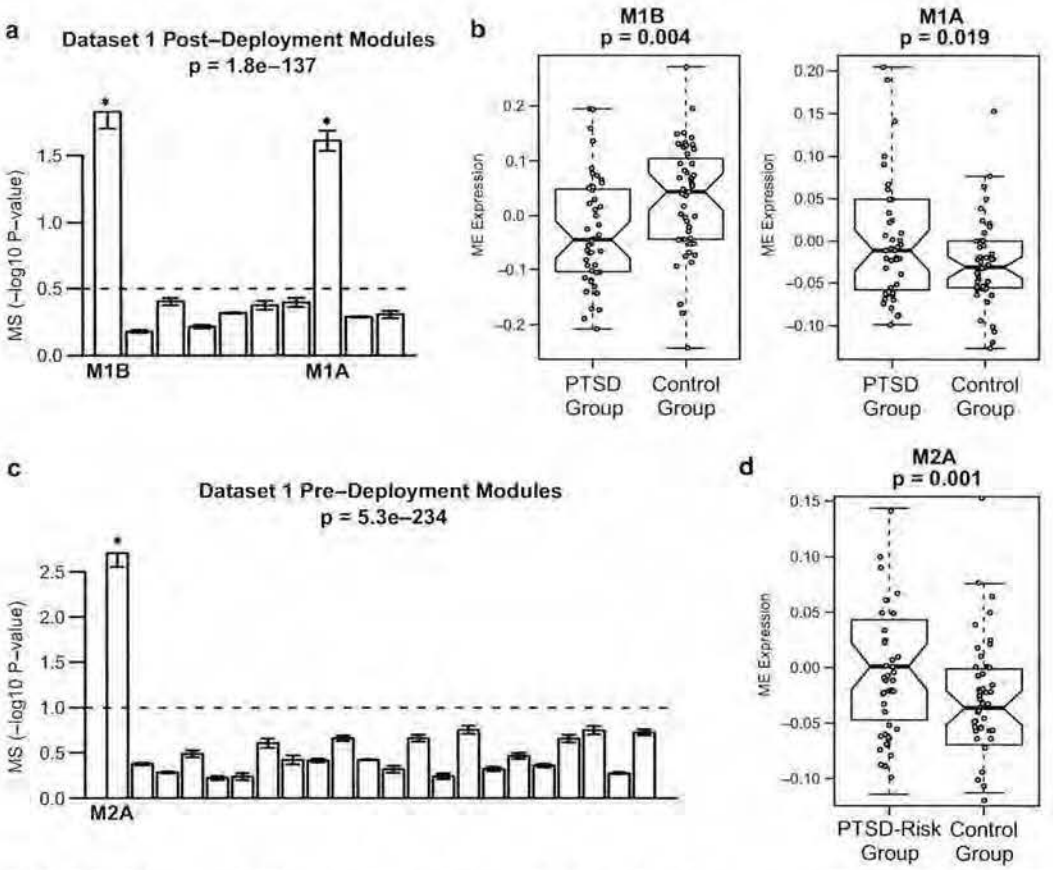


Figure 2. Module significance (MS) and module eigengene (ME) expression boxplots. MS was measured across all pre- and post-deployment modules in Dataset 1. WGCNA detected ten modules post-deployment from a combination of PTSD cases and control (**a**) and twenty-two modules at pre-deployment from a combination of PTSD risk cases and controls (**c**). The y-axis indicates MS by calculating the average $-\log_{10}$ *P*-values, generated by a moderated *t* test, for each gene within a particular module, when assessing differential expression between PTSD cases and controls. Here, a kruskal-wallis *P*-value was used only for descriptive purposes and not inferential. Modules denoted with an asterisk (*) have ME values significantly correlated to conditional states (i.e. PTSD cases or controls). Representative modules with high MS at post-deployment and pre-deployment were investigated for module expression differences. Differences in ME expression were measured using a two-tailed student's *t* test on and a *P*-value < 0.05 is considered significant. Boxplots are displayed for each main group. Significant differences in ME expression were observed in post-deployment modules M1B and M1A (**b**) and in pre-deployment module M2A (**d**).

group ($P = 0.02$, Figure 2b). Subsequently, *ME* values for each module were correlated to all clinical parameters, found in Table 1, to determine module-trait relationships. The *ME* for module M1B was significantly correlated to post-deployment PTSD resilient controls ($r = 0.29$, $P = 0.005$), negatively correlated to post-deployment CAPs and PCL (CAPs, $r = -0.27$, $P = 0.009$; PCL $r = -0.28$, $P = 0.007$) and negatively correlated other measures of CAPS (Supplementary Table 3) but not correlated to any other measured clinical variable, suggesting that differential gene expression in M1B was not confounded by recorded measurements such as body-mass-index, smoking, or alcohol consumption. Genes in M1B were expressed to a greater extent in PTSD resilient controls (Figure 2b) while enrichment analysis revealed a significant association with hemostasis, platelet activation and wound healing (Figure 3a). Further, enrichment for cell-type specificity revealed on over-representation of erythroid expression markers (blood platelets). Hub genes are those most strongly correlated to the *ME* value for a particular module and represent possible disease associated markers,[13] in this case putative PTSD-resiliency markers. The top 5 hub genes in M1B (*C6orf25, CTDSPL, ITGB3, PRKAR2B* and *TUBB1*) were are all associated with hemostasis and in particular, with platelet regulation and function[34–37] (Figure 3b).

The *ME* for module M1A was significantly correlated to PTSD cases ($r = 0.23$, $P = 0.03$), post-deployment CAPs criteria of avoidance (CAPSCA, $r = 0.32$, $P = 0.002$) and post-deployment CAPs criteria of re-experiencing (CAPSBs, $r = 0.2$, $P = 0.05$) but to no other variables (Supplementary Table 3). Genes in M1A were over-expressed in PTSD cases (Figure 2b) while enrichment analysis revealed a significant association with immune response as exemplified by innate responses mediated by interferon (IFN) signalling (Figure 3c), as well as with monocyte specific markers. The top 5 hub genes in M1A included *IFI35, IFIH1, PARP14, RSAD2 and UBE2L6*; all well described interferon stimulated genes[38] and here considered putative PTSD-associated markers (Figure 3d).

Differential module expression pre-deployment in Dataset 1

It is unclear whether the modules identified post-deployment are causal of PTSD development or are simply a consequence of the disorder. To determine if any post-deployment modules could be re-identified and thus associated as causal modules, we constructed a gene co-expression network combining RNA-Seq gene expression data from PTSD-risk cases ($N = 47$) and controls ($N = 47$) pre-deployment in *Dataset 1*. Twenty-two pre-deployment

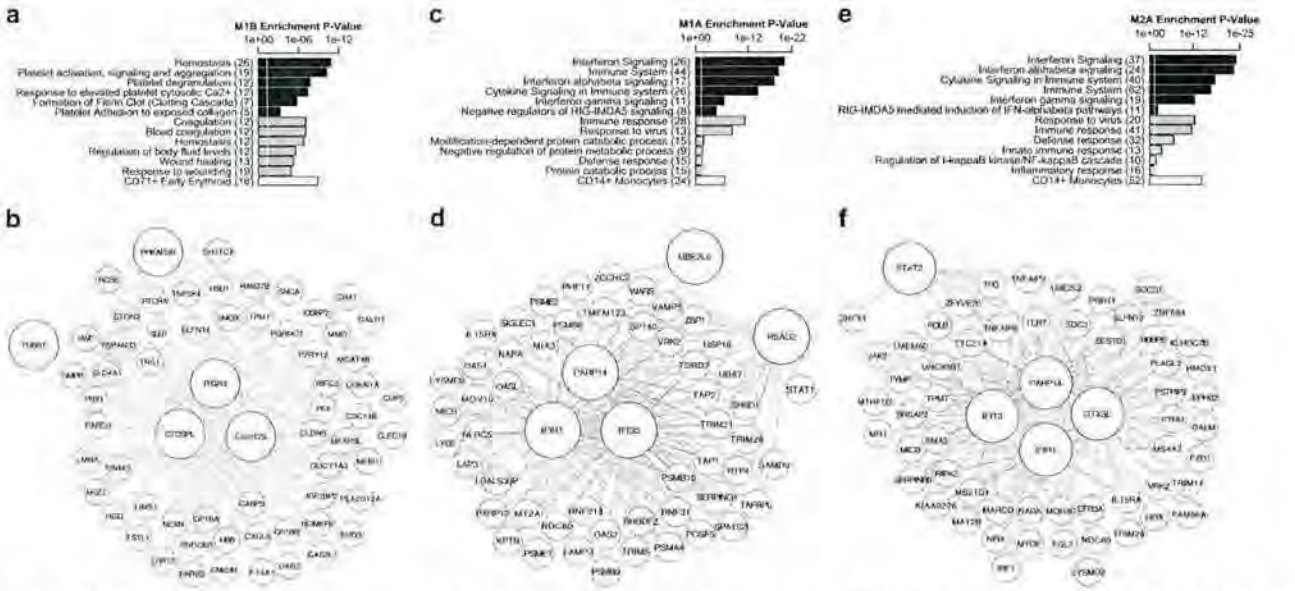


Figure 3. Module characterization for *Dataset 1*. Enrichment analysis and correlation networks for modules M1B (**a** & **b**) and M1A (**c** & **d**) identified post-deployment, and module M2A (**e** & **f**) identified pre-deployment in *Dataset 1*. Enrichment analysis was used to identify the top 6 REACTOME ontology terms (black bars), the top 6 DAVID ontology terms (grey bars) and the most significant cell-type signature (white bar) over-represented in the list of genes within each module. All terms were deemed significant as assessed by a hypergeometric test FDR corrected *P*-value < 0.05 displayed as a white line. The total number of genes within each significant term is denoted within the brackets associated with that term. Gene-networks were constructed selecting the top 150 most significant connections ranked by *kME*. Nodes represent genes and edges represent correlations. The top 5 hub genes, those most correlated to *ME* values, are shown in larger sizes.

modules were identified (fully characterised in Supplementary Table 4) whereby a single module (M2A) was enriched for differentially expressed genes between PTSD-risk participants and controls as reflected by an elevated M5 value (Figure 2c). Along the same lines, M2A module expression was significantly higher in the PTSD risk group ($P=0.001$ and Figure 2d). Module M2A *ME* was significantly correlated to one variable, PTSD-risk ($r=0.32$, $P=0.002$, Supplementary Table 4). Similar to module M1A that was identified post-deployment, enrichment analysis of genes in M2A revealed a significant association with innate immune responses, IFN signalling and monocyte specificity (Figure 3e). The top 5 hub genes were again associated with IFN signalling (*DTX3L, IFIH1, IFIT3, PARP14* and *STAT2)* (Figure 3f). Gene-set overlap analysis compared all of the genes in M2A pre-deployment ($n=245$) to those in M1A post-deployment ($n=115$) to reveal a significant overlap ($\cap=108$, *P=6.7e-181*, Figure 4).

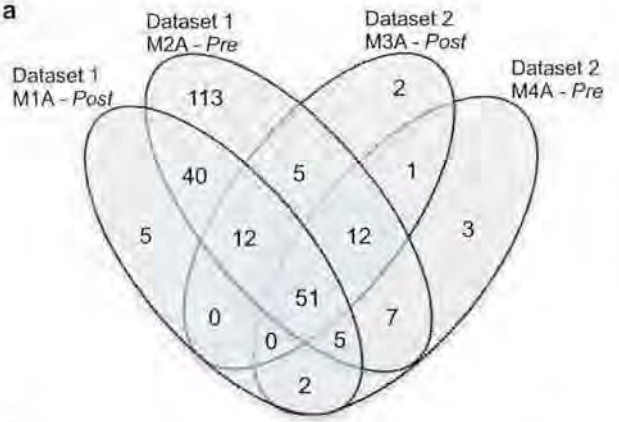
Validation of differential module expression post-deployment in Dataset 2

To validate post-deployment findings in *Dataset 1* we assessed *Dataset 2* for similar network properties in a combined network analysis of PTSD cases ($N=24$) and controls ($N=24$) post-deployment. Out of 8 modules (full characterisation Supplementary Table 5), a single module (M3A) contained an enrichment of differentially expressed genes (Supplementary Figure 2A) demonstrating a modest, yet insignificant, increase in module expression within the PTSD group ($P=0.1$, Supplementary Figure 2B). The *ME* was significantly correlated to post battle experience ($r=0.4$, $P=0.004$), post-deployment CAPS ($r=0.32$, $P=0.03$) and weakly correlated to a PTSD cases ($r=0.21$, $P=0.1$, Supplementary Table 5). The genes in this module were over-expressed in PTSD cases relative to controls (Supplementary Figure 2B) and enrichment analysis revealed a significant association with innate immune responses, IFN signalling and monocytes (Supplementary Figure 3A). The top 5 hub genes (*DDX58, IFI35, IFIT5, PARP9 and ZBP1*) were again all associated with IFN signalling (Supplementary Figure 3B). A highly significant overlap in post-deployment module genes across M1A ($n=115$) in *Dataset 1* and M3A ($n=83$) in *Dataset 2* ($\cap=63$, $P=2.0E-105$, Figure 4b) confirmed the identification of a dysregulated innate immune module related to PTSD cases across two independent datasets.

Validation of differential module expression pre-deployment in Dataset 2

To re-confirm pre-deployment findings from *Dataset 1*, PTSD-risk cases ($N=24$) and controls ($N=24$) pre-deployment were combined from *Dataset 2* and subjected to network analysis which identified 11 modules (full characterisation in Supplementary Table 6). A single module (M4A) was enriched for differentially expressed genes between PTSD-risk cases and controls (Supplementary Figure 2C). The PTSD-risk group displayed a significant over-expression of module expression ($P=0.01$, Supplementary Figure 2D). The *ME* for M4A was significantly correlated to PTSD-risk ($r=0.36$, $P=0.01$) and CAPs ($r=0.44$, $P=0.002$, Supplementary Table 6). Moreover, enrichment analysis of M4A revealed a significant association with innate immune responses, IFN signalling and monocytes (Supplementary Figure 3C), and the top 5 hub genes (*PARP9, UBE2L6, STAT2, TRIM22* and *GBP1*) were again all associated with IFN signalling (Supplementary Figure 3D). All pairwise gene-set overlap analyses across modules M1A, M2A, M3A and M4A revealed a highly significant overlap (Figure 4b) and hub gene expression for these modules showed elevated expression in PTSD groups when compared to controls both pre- and post-deployment across both datasets (Supplementary Figure 4). These results demonstrate the association of a dysregulated innate immune module, related to IFN signalling, which appears to define at least part of the pathophysiology of PTSD through causal association to PTSD development.

a



b

	M1A	M2A	M3A	M4A
M1A	-	*6.7E-181*	*2.0E-105*	*1.0E-134*
M2A	**108** ∩	-	*6.3E-134*	*2.4E-121*
M3A	**63** ∩	**80** ∩	-	*8.8E-152*
M4A	**58** ∩	**75** ∩	**69** ∩	-

c

Gene Symbol	kME Rank M1A	M2A	M3A	M4A
IFIH1	**3**	**1**	12	**7**
STAT2	**6**	**2**	19	**3**
PARP14	**4**	**3**	22	40
DTX3L	39	**4**	46	39
IFIT3	**10**	**5**	**8**	**10**
IFI35	**2**	**6**	**4**	52
UBE2L6	**1**	**7**	18	**2**
PARP9	47	**8**	**2**	**1**
TRIM22	31	**9**	14	**4**
DDX58	36	**10**	**3**	26
TRIM5	34	11	41	16
CMPK2	34	12	51	34
IFIT5	17	13	**1**	21
RSAD2	**5**	14	35	48
HERC5	11	15	17	14
IFI6	13	17	32	43
OAS3	15	18	15	44
IRF9	25	19	44	62
IFIT2	37	20	24	45
IFIT1	16	25	26	49
SERPING1	30	26	34	**6**
STAT1	21	27	30	23
GBP1	43	28	**10**	**5**
IFI44	24	32	**9**	20
SAMD9L	14	33	13	12
PML	**8**	34	28	32
Continued...				

Continued... Gene Symbol	M1A	M2A	M3A	M4A
ZBP1	51	35	**5**	38
APOL6	31	36	36	11
APOL1	30	37	67	75
MX1	38	38	**7**	37
IFI44L	28	39	58	65
DDX60	37	40	16	28
BATF2	32	43	83	50
OASL	40	44	62	69
EPSTI1	40	45	42	25
FBXO6	42	47	56	15
LAP3	70	50	11	41
OAS2	46	52	21	18
TAP1	63	58	31	**8**
PARP12	33	63	23	27
RTP4	55	65	27	13
TAP2	45	67	53	70
SPATS2L	67	69	64	81
CXCL10	35	70	57	68
LY6E	65	75	37	64
OAS1	74	84	43	66
DHX58	38	93	47	29
USP18	66	97	82	82
CD274	33	121	55	30
MOV10	94	123	60	31
ETV7	41	128	79	74
GBP5	44	151	38	**9**

Figure 4. Venn Diagram of Innate Immune Modules across *Dataset 1* and *Dataset 2*. Venn Diagram (**a**) depicting significant overlap in genes belonging to modules M1A post-deployment and M2A pre-deployment in *Dataset 1* as well as modules M3A post-deployment and M4A pre-deployment in *Dataset 2*. Gene overlap (∩) with associated hypergeometric *P*-value, in italics, are depicted for all pairwise comparisons of module genes (**b**). The overlap identified 51 genes found across all four analyses (**c**) which are displayed in the table along with the corresponding kME rank (i.e. rank of connectivity) for each gene within a particular module. A high rank indicates hub gene status (i.e. PTSD risk and PTSD associated markers). Numbers in bold outline the top 10 hub genes across each module, respectively. Genes are ordered accordingly to M2A kME. All 51 genes are displayed via heatmap in Supplementary Figure 4.

DISCUSSION

We investigated the high-order system-level properties of PTSD using an unsupervised network-based approach (WGCNA) to identify differences at the gene co-expression level, rather than investigating at the individual gene level. Gene expression data were generated by RNA-Seq (*Dataset 1*) and microarray (*Dataset 2*) using PBL samples isolated from U.S. Marines pre- and post-deployment to conflict zones (i.e. Iraq and Afghanistan). Our comprehensive and prospective experimental design allowed the investigation of both biological processes that define PTSD and those driving the development of this disorder, and further, allowed the re-confirmation of findings in an independent dataset. This is the first time dysregulated gene networks specific for innate immunity have been used to characterise causal and consequential molecular signatures of PTSD and then to further replicated these findings across independent datasets.

A novel finding from our network analyses was the identification of modules related to hemostasis and wound responsiveness expressed to a greater extent post-deployment in US Marines who did not develop PTSD (Figure 2b), as in module M1B (Figure 3a). Interestingly, the three other network analyses also detected modules related to hemostasis and wound response with significant overlap (M16 pre-deployment *Dataset 1*; M7 and M6 indented post- and pre-deployment in *Dataset 2*; Supplementary Figure 5, Supplementary Tables 4). These other modules revealed patterns of heterogeneous gene expression irrespective of group status and time-point suggesting that these modules and corresponding processes may infer wound resilience in only a small subset of individuals. Along these lines, it has been well documented that different degrees of stress will elicit different stress responses (review[39]), and in particular, a response involving blood platelets, has been shown to be a critical biomarker of hemostatic, thrombotic, and inflammatory challenges to an organism and a key player in cardiovascular disease and chronic based stress, as in PTSD.[40,41] Moreover, in a review of a large number of studies examining various tissue types, it was found that different types of psychological stress were associated with impaired wound healing.[42] A meta-analysis found an inverse correlation ($r=-0.42$) between psychological stress and wound healing[43] supporting the positive association between wound healing and PTSD resilience ($r=0.29$, $P=0.005$) found in this study. This suggests that high levels of stress may hinder proper wound healing during/after battlefield trauma, although the degree of such stress appears to be a key factor for establishing associations with the hemostatic system.

Our central finding was the identification of a dysregulated innate immune module associated with the development of PTSD (Figures 2 and 3, Supplementary Figure 3), illuminated by the replication of modules post-deployment (M1A and M3A) and those pre-deployment (M2A and M4A) that could be associated with PTSD. These findings suggest that differences in innate immunity modules were not simply a consequence of the PTSD state post-deployment but also have causal relevance for PTSD development and explain at least part of the pathophysiology of the disorder, exemplified by their identification pre-deployment. These results highlight our differential expression analyses (Supplementary Figure 1) and our previous reports of C-reactive protein (CRP), a general marker of immune activation and inflammation, and 5′-oligoadenylate synthetase genes (*i.e. OAS1, OAS2, OAS3*) as markers of the antiviral interferon response, that were associated with an increased risk of developing PTSD.[44,12] However, our current findings dramatically extend these results by showing that the IFN response is being modulated to a much greater extent than previously thought pre- and post-deployment. Notably, a number of single case studies have reported that treatment of hepatitis C virus (HCV) infected PTSD subjects with recombinant interferon (IFN- α2b) precipitated PTSD symptoms.[45,46] In our study, where subjects were not receiving IFN therapy, it is unclear what is stimulating the IFN response.

Our observations lead to several fundamental questions and some putative solutions. First, how does one interpret the over-

expression of innate immunity genes found prior-to trauma? One possible explanation is that both acute and severe stress, predictors in their own right for PTSD, are also associated with the hyper-activation of the immune system and subsequent inflammation.[47,48] An alternative hypothesis is that stress, pathogens and/or high viral loads may 'prime' the immune system, driving the IFN response, altering a subsequent response to trauma. Along these lines, studies focusing on the gut-brain barrier have shown that intestinal mucosal dysfunction, defined as increased translocation of gram-negative bacteria (leaky gut), plays a role in the inflammatory pathophysiology of depression suggesting that differences in gut flora may stimulate an IFN response.[49] Second, does a dysregulated innate immune module pre-deployment hold predictive value? Our previous work constructing a prognostic classifier from *Dataset 2* pre-deployment participants[12] suggests that immune-related genes do hold predictive value although these results have not yet been replicated across larger datasets using machine-learning methods. Inferring the prognostic relevance of network-based applications remains challenging. However, cross-referencing our findings with this previous work suggests that network statistics, and our innate immune modules, do have potential to contain predictive value. Third, out of the entire network of pairwise correlations between genes across the transcriptome, are the most informative genes interconnected within similar modules or spread out across numerous modules? A possible limitation of this study was that by analyzing co-regulated modules of genes we may have missed individual genes, which do not correlate within our modules of interest although are of functional relevance to PTSD. For example, previous reports specifically target *FKBP5* and *STAT5B* as differentially expressed biomarkers[3–8,11,12] although they were not assigned to co-expressed modules nor found to be significantly differentially expressed between PTSD cases and controls. Finally, of what relevance is PBL gene expression for a disorder primarily associated with the brain? In this study we identify innate immunity and IFN signalling genes whose expression was elevated in PBLs both before and after the development of PTSD (Figure 2 and Supplementary Figure 4). Although the recruitment of such signalling could be triggered by various factors, they ultimately release toxic compounds including degradative enzymes and reactive oxygen species that can impair cellular processes.[50–53] It could be hypothesized that the accumulation of these compounds in the blood prior-to-deployment may be detrimental to the brain if the integrity of the blood-brain-barrier (BBB) was then compromised by injury (e.g. TBI). An increasing body of evidence indicates that changes in the blood may seed pathology in the brain across various disorders. In a recent Multiple Sclerosis study, Minagar and Alexander[54] investigate the association of INF with the BBB suggesting that IFN-γ and other proinflammatory cytokines (TNF-α and IL-1β) disrupt the BBB through a variety of mechanisms. Further, Alzheimer's disease models suggest that breaches in the BBB lead to leakage into the brain of blood-borne molecules that are toxic to neurons and cause neurodegenerative changes.[55] Future studies investigating the role of the BBB in PTSD may provide a detailed explanation for a specific course of PTSD development.

In summary, our data provide a global framework for previously unknown molecular aspects of PTSD and describe a new context concerning the complex pathophysiological nature of PTSD development. Specifically, modules of co-expressed genes associated with the innate immune response and IFN signalling appear to be implicated in the development of PTSD and continue to persist once the disorder is established. Modules associated with hemostasis and wound healing may contribute to resilience against developing PTSD. It is hoped that this study will lead to future work confirming the importance of differences in innate immune factors to the development of PTSD and the role of platelets in the stress response. Ideally, these findings will allow for advanced PTSD detection, which could delay or abrogate PTSD development by identifying susceptible service members prior to deployment to conflict zones by either removing the causal path (i.e. trauma exposure) or through early intervention of new therapies to modulate the interferon signature.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DGB, CN, CHW and DOC obtained the funding for this study. AXM curated clinical information regarding all participants. SJG, DST and SDC generated microarray data. MSB conducted the study which entailed generating RNA-Seq data, writing code for quality testing and computational interrogation of both RNA-Seq and microarray data. MSB drafted and wrote the manuscript with the participation of remaining authors.

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EDITORIAL

New findings from prospective studies

1. Background: the conference

The first biomarker in the military conference, which was a broad discussion of issues in a panel format, was held on September 14, 2012 in New York, NY. The research that was presented is summarized in a paper that includes criteria for biomarkers for PTSD, but with no specific study findings available yet (Lehrner and Yehuda, 2014). This special section covers talks presented at the second military biomarker conference that was held as a satellite to the 43rd meeting of the annual meeting of the Society of Psychoneuroendocrinology. The conference, entitled '*Biomarkers in the Military*' was held at the Royal Marine Base in Amsterdam August 23, 2013. The aim of the satellite was to bring together researchers supported by Departments of Defense, Veterans Administrations, National Institutes of Health, and other agencies around the world engaged in study of biomarkers in the military. This special section, the first to assemble new findings focused on biomarker discovery, presents work of researchers from a number of North Atlantic Treaty Organization (NATO) partners collaborating in the International Security Assistant Force (ISAF). While one paper is a pre-clinical study relevant to biomarker discovery, most are clinical. The work, as described below, includes original contributions from the gold-standard study design, prospective longitudinal studies as well as from cross-sectional research.

2. Special section papers

Schmidt et al. provide a literature review and conceptual framework for prospective longitudinal studies. They review progress in the search for PTSD risk and resiliency biomarkers in both civilian and military studies. Despite a significant increase in the number of prospective trials over last couple of years and some promising results, Schmidt et al. address the need for well-designed pre-post studies. In their rigorous selection of over 8,000 papers targeting PTSD biomarker research they could only include 9 imaging and 27 molecular studies that hold power for biomarker identification. They underscore the increasing evidence that polymorphisms of HPA axis associated genes interact with early life stress to enhance the vulnerability for adulthood PTSD. Yet, none of the proposed PTSD risk markers is currently clinically applicable since all proposed markers lack PTSD specificity.

Nievergelt and colleagues present the first ever genome-wide association study (GWAS) in a military cohort. This cohort, the Marine Resiliency Study (MRS) cohort was designed as a prospective study to determine risk and resilience genes by analyzing genes from active duty personnel about to deploy to Iraq and Afghanistan (Baker et al., 2012). Because the intention was to follow nearly 3500 troops when they returned from combat, the study offered the possibility to determine whether information about GWAS and other markers predicted short- and long-term post-combat mental and physical outcomes. The study by Nievergelt et al. is also noteworthy for being the first multi-ethnic/racial GWAS of PTSD, and thus highlights the potential to increase power through meta-analyses across ancestry groups. In this initial analysis of the data, Nievergelt and colleagues identified the phosphoribosyl transferase domain containing 1 gene (PRTFDC1) as a genome-wide significant PTSD locus, with a similar effect across ancestry groups. Another key finding of the paper is that a cross-disorder polygenic analysis shows the existence of common SNPs between posttraumatic stress disorder and bipolar disorder. By seeking data from other studies to locate replication cohorts, the study also highlights important strategies for interpreting similarities and differences between military and other samples.

Another paper, by Tylee et al. presents data from the same cohort, the MRS study, building upon earlier work (Glatt et al., 2013). It provides preliminary results of proof-of-principle findings for a diagnostic blood-based mRNA-expression biomarker panel in PTSD based on gene-expression levels in peripheral blood samples. The authors present a prospective study in 50 U.S. Marines (25 eventual PTSD cases and 25 non-PTSD comparison subjects) with data gathered prior to their deployment overseas to war-zones in Iraq or Afghanistan, and again upon return. Their panel of biomarkers in peripheral blood cells of eventual PTSD cases

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was significantly enriched for immune genes, and achieved 70% prediction accuracy in an independent sample based on the expression of 23 full-length transcripts, and attained 80% accuracy in an independent sample based on the expression of one exon from each of five genes.

From the same research group (Marine Resiliency Study II; Neurocognition project), Risbrough et al., analyzing data from Marines bound for Afghanistan prior to their deployment, uses a functional biomarker approach to assess the effectiveness of the fear potentiated startle paradigm in producing fear learning and extinction, and the association of performance with baseline psychiatric symptom classes. Comparison of four groups (Healthy, PTSD symptoms, Anxiety symptoms, and Depression symptoms) across the cohort shows differential patterns of fear conditioning and extinction, with the PTSD symptom group, in contrast to anxiety, depression and healthy showing reduced fear inhibition, consistent with the idea that safety signal discrimination is a relatively specific marker of PTSD. The researchers plan to follow up to determine if deficits in fear inhibition vs. exaggerated fear responding are separate biological 'domains' that might predict differential biological mechanisms and possibly treatment needs, as well as to pursue longitudinal analyses to examine whether poor safety signal learning provides a marker of vulnerability to develop PTSD or is specific to symptom state.

Four papers are driven from data from a prospective longitudinal study in Dutch soldiers deployed to Afghanistan as part of ISAF, called Prospective Research in Stress related Military Operations (PRISMO). Acquisition of biological samples for this study started in 2005 and lasted until 2008 and included a total of 1032 soldiers. In the first 2 year follow-up prevalences of mental health symptoms do not differ much from those reported by other NATO partners (Reijnen et al., 2014). The design allows for identification of blood-based biomarkers, (epi)genetic analyses and symptom trajectories as this cohort is being followed up at multiple time points up to 10 years post deployment. A small group has been scanned with functional neuroimaging of the brain before and after deployment driving new findings e.g. on the role of the amygdala and glucocorticoid receptor number (Geuze et al., 2012; van Wingen et al., 2011). This special issue contains four studies by Boks et al., van Zuiden et al, Reijnen et al, and Smid et al., published from this cohort. The first, by Boks et al. focuses on epigenetic age. It has been suggested that traumatic stress has an impact on aging at the cellular level, which can be investigated by estimating epigenetic age based on DNA methylation profiles. While our prevailing understanding is that a telomere shortening is associated with PTSD or PTSD onset, Boks et al. found a remarkable acceleration of aging induced by combat exposure. Development of initial PTSD symptoms (at 6 months) was associated with telomere lengthening and reversed epigenetic aging which may be best understood to be linked to a dysfunctional compensatory cellular aging reversal in early stages of PTSD. The second paper, by Van Zuiden et al. followed up on prior findings of higher pre-deployment GR number in PBMCs in soldiers who developed high levels of PTSD symptoms after deployment. In the current analysis it was demonstrated that the differences in the peripheral GC-sensitivity persisted until at least 6 months after return from combat. This could indicate that in vitro GC-sensitivity of T-cells and monocytes represents a persistent biological vulnerability factor for development of PTSD. The third paper, by Reijnen et al. added evidence for a role of the Hypothalamic Pituitary Gonadal (HPG) axis for the development of PTSD. The HPG axis parameter testosterone was analyzed in the total sample of deployed soldiers. Pre-deployment testosterone levels predicted the development of PTSD symptoms at 1 and 2 years post-deployment, with alterations in testosterone levels shortly after deployment not being predictive, but the pre-deployment testosterone levels at longer post-deployment timeframes being associated with PTSD. Lastly, Smid et al. followed up on earlier work on the model of stress sensitization that was previously validated in the PRISMO cohort (Smid et al., 2013). Especially in high combat exposed soldiers in the first 6 months after combat a higher T-cell chemokine production was associated with increases in PTSD symptoms. An interesting interaction between cytokines and stressful life effects at homecoming was associated with changes in PTSD symptoms. As mitogen-induced cytokine and chemokine production constitute markers of stress sensitization this finding may imply that efforts to prevent progression of posttraumatic distress should aim at creating a 'comfort zone', by keeping the highly exposed veterans in the first couple of months after homecoming safe, away from unnecessary stressors, thus preventing stress sensitization.

In the only preclinical paper Rutten et al. studied the effects of 10 days of social defeat stress on behavior and Dnmt3a expression in relation to neurogenesis in the mouse hippocampus. Mice resilient to defeat stress show higher Dnmt3 expression compared to controls (non-defeat) as well as to susceptible groups. It is known that epigenetic modifications, such as DNA methylation, can occur in response to environmental influences to alter the functional expression of genes. This study adds preclinical evidence of the role of DNA methylation in susceptibility to severe stressors. These findings provide a pre-clinical scientific foundation for the assessment of the impact of trauma exposure on DNA methylation e.g. in prospectively followed military cohorts.

Two additional papers used a cross-sectional approach. In a search for the relation between inflammatory markers and brain integrity O'Donovan et al. looked at a large cohort of Gulf war veterans for associations between peripheral inflammatory markers and brain integrity, in particular hippocampal volume. Specific inflammatory signaling proteins (sTNF-RII, but not IL-6) were significantly associated with reduced hippocampal volume and PTSD symptoms. In a small sample Yehuda et al. presented results of a new developing method of DTI tractography data from 20 Gulf War veterans. Their observations are consistent with a functional model that converges on the concept of increased amygdala responsivity in association with anterior cingulate modulation in PTSD.

Another set of two papers focuses on the endocannabinoid system. It is only recently that researchers embrace the old notion that cannabis has qualities that are favored by PTSD patients. Neumeister et al. reviewed translational evidence for a role of endocannabinoids in the etiology and treatment of PTSD. Multiple studies are reviewed that report reduced endocannabinoid availability and elevated cannabinoid type 1 (CB1) receptor availability in PTSD and its link

to abnormal threat processing and anxious arousal symptoms. This study lays the foundation for a mechanism-based pharmacotherapy approach by Jetly et al. This group conducted a small randomized clinical trial (RCT) with a double placebo controlled cross-over design in a Canadian military personal suffering from PTSD. In a brief report they demonstrated good efficacy for Nabilone, specifically for trauma related nightmares, thus adding support for the potential use of synthetic cannabinoids.

As the last paper in this special section, Yehuda et al. employed the model of exposure based 'golden hour' opportunities (Vermetten et al., 2014b) by augmenting psychological treatment with medication in veterans with PTSD. They performed a highly important pilot study investigating the potential therapeutic benefit of hydrocortisone augmentation of prolonged exposure therapy for combat veterans with PTSD. Hydrocortisone augmentation was associated with greater reduction in total PTSD symptoms compared to placebo. Moreover, the biological data demonstrated an impressive correlation between glucocorticoid sensitivity and CAPS total score in hydrocortisone recipients. Their finding of a significant baseline difference in glucocorticoid sensitivity between responders and non-responders is also very relevant for future investigations into the fundamental neurobiological mechanisms underlying the pathophysiology of PTSD.

3. Promises

As the papers illustrate there has been an enormous effort to capture risk and resilience factors, as well as to identify biomarkers of expressed illness. Various Departments of Defense (DOD) around the world have, and continue to invest significant resources to augment force protection and security by seeking methods to optimize prevention and treatment strategies for behavioral and mental health problems. We are grateful for the military leadership in their foresight and support of this research that enables a wide range of researchers across the globe to collaborate and to move the field forward. Given the similarities in deployment-related mental health support across nations (Vermetten et al., 2014a), collaborative efforts can be entertained that can enable both replication as well as validation of biomarker findings. We are grateful for so much support in organizing this satellite, dissemination of this research and promoting that these efforts are sustained. Lastly, a special thanks to all the reviewers for this special section. We are advancing rapidly, but these efforts will need to be sustained over the next decades as we consolidate knowledge in the field.

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Original Investigation

Association of Predeployment Heart Rate Variability With Risk of Postdeployment Posttraumatic Stress Disorder in Active-Duty Marines

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Editorial page 964

Supplemental content at jamapsychiatry.com

IMPORTANCE Disrupted autonomic nervous system functioning as measured by heart rate variability (HRV) has been associated with posttraumatic stress disorder (PTSD). It is not clear, however, whether reduced HRV before trauma exposure contributes to the risk for development of PTSD.

OBJECTIVE To examine whether HRV before combat deployment is associated with increased risk of a PTSD diagnosis after deployment when accounting for deployment-related combat exposure.

DESIGN, SETTING, AND PARTICIPANTS Between July 14, 2008, and May 24, 2012, active-duty Marines were assessed 1 to 2 months before a combat deployment and again 4 to 6 months after their return. The first phase of the Marine Resiliency Study (MRS-I) included 1415 male Marines, 59 of whom developed PTSD after deployment. Participants in the second phase of the Marine Resiliency Study (MRS-II) included 745 male Marines, 25 of whom developed PTSD after deployment. Analysis was conducted from November 25, 2013, to April 16, 2015.

MAIN OUTCOMES AND MEASURES Predeployment HRV was measured via finger photoplethysmography during a 5-minute period of rest. Frequency-domain measures of HRV were generated. Diagnosis of PTSD was determined using the Clinician-Administered PTSD Scale.

RESULTS After accounting for deployment-related combat exposure, lower HRV before deployment as measured by an increased low-frequency (LF) to high-frequency (HF) ratio of HRV was associated with risk of PTSD diagnosis after deployment (combined MRS-I and MRS-II cohort meta-analysis odds ratio, 1.47; 95% CI, 1.10-1.98; $P = .01$). The prevalence of postdeployment PTSD was higher in participants with high predeployment LF:HF ratios (15.8% [6 of 38 participants]) compared with participants who did not have high LF:HF ratios (3.7% [78 of 2122 participants]).

CONCLUSIONS AND RELEVANCE This prospective longitudinal study provides initial and modest evidence that an altered state of autonomic nervous system functioning contributes to PTSD vulnerability, taking into account other key risk factors. If these findings are replicated, interventions that change autonomic nervous system function may open novel opportunities for prevention and treatment of PTSD.

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Posttraumatic stress disorder (PTSD) is, historically and currently, a significant public health problem in individuals deployed to war. Lifetime prevalence of the disorder is approximately 19% in Vietnam-era combat veterans[1] and 13% to 15% in US military servicemembers serving in this era's conflicts in Iraq and Afghanistan[2,3] compared with the 8% general prevalence rate of PTSD in the United States.[4] These differences in prevalence rates may be in part attributable to variations in the disorder's diagnostic criteria or how it is assessed. Regardless, psychological and functional consequences of PTSD can be devastating (eg, high suicide rates[5] and long-term disability with substantial impairment in functioning[6]). Furthermore, PTSD is associated with several adverse health consequences.[7,8]

Heart rate variability (HRV) is the quantitative assessment of variation in heartbeat intervals and is a sensitive index of autonomic nervous system (ANS) function.[9] Heart rate is modulated by both the parasympathetic and sympathetic branches of the ANS via influences on the sinoatrial node pacemaker.[10] The consistent findings of reduced HRV in PTSD suggest autonomic inflexibility due to sympathetic overactivity and/or parasympathetic insufficiency,[11-13] potentially mediated by the presence or worsening of the cardiovascular problems that are common in the disorder.[14] It is unclear whether autonomic inflexibility is simply reduced during active PTSD symptoms or whether abnormalities can also be detected in individuals who are at risk for PTSD. In other words, does diminished HRV before trauma increase the likelihood of stress disorder symptoms after trauma? Low parasympathetic control of sympathetic output could reflect an at-risk state for development of stress disorders via reduced cortical modulation of ANS responses to stress[15,16] or, alternatively, could be a traitlike phenomenon associated with decreased resilience to stress. Some evidence[17] suggests that diminished HRV immediately after trauma can predict development of PTSD; however, to our knowledge, HRV before trauma has not been tested. Identifying biology-based markers of PTSD susceptibility will enable delineation of mechanisms that confer susceptibility to the long-term effects of trauma and inform preventive strategies.

To this end, we tested the hypothesis that HRV before trauma is associated with the development of PTSD in a large group of Marines and Sailors after their return from deployment to a combat zone. Active-duty Marines in the Marine Resiliency Study (MRS), previously described by our group,[13,18-20] were evaluated for HRV and PTSD symptoms before and after deployment. We[19] reported a cross-sectional association before deployment between reduced HRV and PTSD in the MRS cohort. In the present study, we tested our hypothesis that low HRV is a risk factor for PTSD, predicting that Marines and Sailors who developed PTSD after deployment would exhibit low HRV before deployment.

Methods

Participants

Participants were active-duty US Marines and Sailors tested approximately 1 month before deployment to Iraq or Afghanistan as part of the first phase of the MRS (MRS-I), a prospective longitudinal study to examine markers of risk and resilience to combat stress. The participants were reassessed approximately 3 and 6 months after return from deployment. The 6-month time frame was the focus of the present study in an effort to assess the prolonged PTSD syndrome. Four infantry battalions were tested between July 14, 2008, and May 24, 2012, at 1 of 2 bases in Southern California.[18] Additional data from a smaller cohort (drawn from the second phase of the MRS [MRS-II]) were analyzed separately owing to assessment time differences (assessments 1 week before deployment and 4-5 months after deployment, with the final evaluation occurring October 10, 2013). Studies were approved by the institutional review boards of the Veteran's Administration San Diego Healthcare System; the University of California, San Diego; and the Naval Health Research Center. All participants provided voluntary written informed consent. Data analysis was conducted between November 25, 2013, and April 16, 2015.

All active-duty Marines planning to deploy with their units were considered for study inclusion. Women were not included since female Marines were not part of infantry battalions at the time of testing.

Procedure

All participants from whom blood samples were drawn received a nominal financial compensation. The predeployment test battery included a comprehensive evaluation of demographic information, history, and current symptoms with respect to military service; drug, alcohol, and tobacco use; psychiatric conditions; head injuries; and psychological trauma.[18] Blood samples were used to determine genetic-based ancestry information (eMethods in the Supplement).[21] The postdeployment test battery was similar in duration and included reevaluation of symptoms and psychological adjustment as well as deployment-related occurrence of traumatic brain injury.

To measure HRV, participants were seated in quiet rooms and a finger photoplethysmograph ([PPG] Pasco Scientific) was placed on the nail of the right fifth finger. The PPG is an optical technique used to detect beat-to-beat blood volume changes in microvascular tissue and was sampled at 1000 Hz (eMethods in the Supplement).[19] We elected to use a 5-minute at-rest PPG reading because this method offers cost-effectiveness, rapid implementation, and feasibility for screening large numbers of people compared with electrocardiogram protocols. However, PPG is limited in that it cannot accurately detect respiration rate and the time frame is not adequate to measure very low frequency components of the spectral band.

Outcome Measurements

The PPG data were processed to generate HRV variables using our group's published methods.[19] Frequency-domain HRV measures were generated per the recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology.[22] The low-frequency (LF) and high-frequency (HF) components and the LF:HF ratio were examined (eMethods in the Supplement).

The Clinician-Administered PTSD Scale (CAPS)[23] was used to determine the presence of a PTSD diagnosis at the prede-

ployment and postdeployment (6-month) visits. The CAPS is a structured interview and is considered the criterion standard for ascertainment of a PTSD diagnosis using *DSM-IV* criteria. Using CAPS responses, we categorized participants as either not meeting or meeting criteria for PTSD at each of the 2 time points. Criteria were derived from the *DSM-IV*: at least 1 B symptom (traumatic event is persistently reexperienced), 3 C symptoms (persistent avoidance of trauma-associated stimuli and numbing of general responsiveness), and 2 D symptoms (persistent arousal symptoms). Because we used a *DSM-5* diagnosis approach with the CAPS, there was not a quantitative criterion for a minimum CAPS score. The range of CAPS scores for participants who met criteria for PTSD was 28 to 96; the mean (SD) score was 61.91 (16.07).

Statistical Analysis

The LF, HF, and LF:HF ratio were positively skewed and natural log transformations were generated; such transformations are widely used in HRV research.[12,13,24-27] After transformation, outliers greater than 3 SDs from the mean were excluded from subsequent data analyses,[28] and data were reinspected for normal distribution.

To assess the relationship between HRV before deployment and PTSD at the postdeployment visit, multivariate logistic regressions were conducted for each of the 3 HRV variables for the MRS-I and MRS-II cohorts. The outcome variable was a comparison of participants who did not meet criteria for PTSD either before or after deployment with participants who did not meet PTSD criteria before deployment but fulfilled diagnostic criteria for the disorder after deployment. A summary measure of combat exposure and deployment-related stressors was included as a covariate in the regression model since this factor is the strongest causal predictor of combat-related PTSD. This factor was derived from the Deployment Risk and Resilience Inventory (DRRI).[29] Four DRRI subscales were combined into 1 composite score to measure deployment stressors: Combat Experiences, Aftermath of Battle, Deployment Concerns About Life and Family Disruptions, and the Difficulty Living and Working Environments (eMethods in the Supplement).

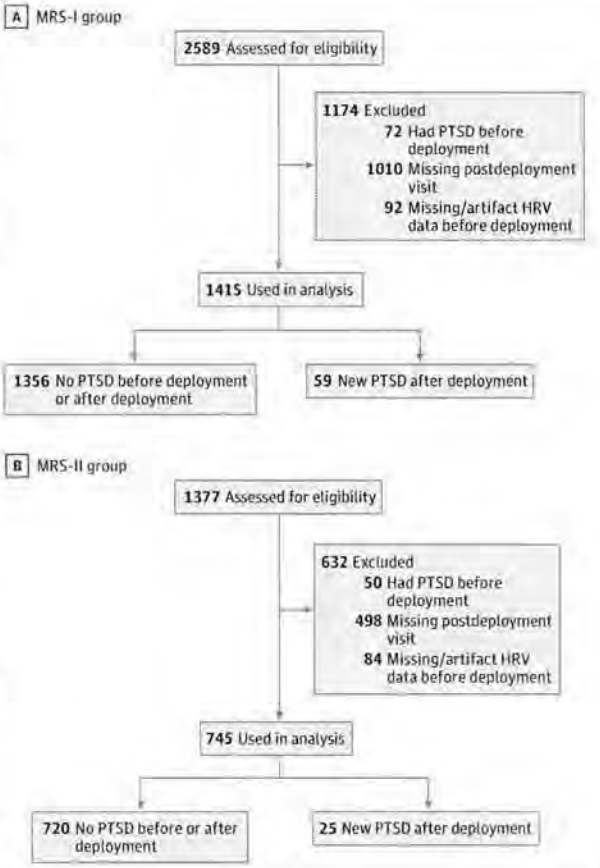
The MRS-I and MRS-II analyses were next subjected to a fixed-effect meta-analysis to generate an overall effect size. Power to detect a significant effect was calculated using the a priori power analysis method within the logistic regression module of G*Power, version 3.1.9.[30]

To determine whether HRV remained significant in the regression model when accounting for other variables that have been closely associated with HRV and/or PTSD in our[19] and others'[11-13,31] findings, the HRV variable that achieved significance in the primary regressions was reassessed in an additional multivariate logistic regression controlling for the following covariates: age, ancestry, battalion, CAPS total scores at the predeployment visit, deployment-related traumatic brain injury as defined by a self-report of a new head injury sustained during deployment that was accompanied by either a loss of consciousness or altered mental status, and DRRI scores.

Post hoc correlations between HRV variables and clinical variables were conducted using Pearson *R* correlation coefficients. A substantial proportion of participants (464 of 2160 [21.5%]) had a CAPS total score of zero at the postdeployment visit. Those participants' data were not included in the correlational analysis.

Significance levels were set at $P < .02$ (χ^2 analysis) for the primary regressions to account for multiple comparisons (3 HRV variables). Significance levels for the follow-up regression using the single HRV variable were set at $P < .05$. Effect sizes were calculated when relevant (eg, odds ratios [ORs] for regression analyses). Statistical analyses were conducted with SPSS, version 20 (SPSS Inc), and *R*, version 2.15.3 (R Foundation for Statistical Computing).

Figure 1. Marine Resiliency Study (MRS)-I and MRS-II Participant Flow Diagrams



HRV indicates heart rate variability; PTSD, posttraumatic stress disorder.

Results

The overall demographic composition of Marines and Sailors in the MRS has been reported.[18-20,32] Overall, the analyses suggested that lower HRV at the predeployment visit, as measured by higher values of the LF:HF ratio, was associated with increased risk of a PTSD diagnosis at the postdeployment visit. Participants with high LF:HF ratios at the predeployment visit

Table 1. Demographic and Descriptive Information for Participants in the MRS

Characteristic	MRS Phase	Mean (SD) [No.]	Statistical Value
MRS-I, No.		1415	
MRS-II, No.		745	
Age, y	I	22.4 (3.5)	Mann-Whitney = 487 320.50; P = .002
	II	21.9 (2.8)	
Ancestry, No. (%)	I	European American, 903 (63.8) African American, 83 (5.9) Hispanic/Native American, 253 (17.9) Asian/other, 176 (12.4)	χ^2 = 1.80;P = .62
	II	European American, 479 (64.3) African American, 39 (5.2) Hispanic/Native American, 145 (19.5) Asian/other, 82 (11.0)	
Prior deployment, No. (%)	I	570 (40.3)	χ^2 = 21.76;P < .001
	II	379 (50.9)	
Time in the military before deployment, mo	I	31.6 (35.1)	Mann-Whitney = 522 321.50; P = .61
	II	29.4 (25.5)	
AUDIT total score before deployment	I	7.4 (6.6)	Mann-Whitney = 520 056.50; P = .92
	II	6.9 (5.5)	
Hours since caffeine use before deployment[a]	I	6.4 (6.3) [799]	Mann-Whitney = 164 654.00; P = .004
	II	7.8 (7.2) [457]	
Hours since nicotine use before deployment[a]	I	3.1 (3.8) [654]	Mann-Whitney = 113 119.50; P < .001
	II	4.0 (4.5) [396]	
CAPS total score			
Before deployment	I	13.0 (12.0)	Mann-Whitney = 480 851.50; P < .001
	II	11.1 (10.8)	
After deployment	I	14.6 (16.4)	Mann-Whitney = 503 461.00; P = .05
	II	15.3 (15.7)	
Sustained TBI during deployment, No. (%)	I	274 (19.4)	χ^2 = 0.07;P = .82
	II	148 (19.9)	
DRRI composite imputed score after deployment[b]	I	−0.09 (0.79)	Mann-Whitney = 481 285.00; P < .001
	II	0.01 (0.77)	

Abbreviations: AUDIT, Alcohol Use Disorders Identification Test; CAPS, Clinician-Administered PTSD Scale; DRRI, Deployment Risk and Resilience Inventory; MRS, Marine Resiliency Study; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a] Calculated only in participants who self-reported use of this substance within 24 hours.

[b] Imputed composite score as described in the eMethods in the Supplement.

Table 2. Parameter Estimates for Multivariate Logistic Regressions Including the LF:HF Ratio in Predicting PTSD After Deployment

	MRS-I[a]			MRS-II[b]			Meta-analysis		
Characteristic	OR (95% CI)	Wald χ^2	P Value	OR (95% CI)	Wald χ^2	P Value	OR (95% CI)	Wald χ^2	P Value
DRRI after deployment	2.95 (2.15-4.09)	43.39	<.001	2.55 (1.50-4.43)	11.59	.001	2.84 (2.15-3.74)	54.75	<.001
LF:HF ratio[c]	1.63 (1.14-2.34)	7.07	.008	1.20 (0.72-2.03)	0.45	.50	1.47 (1.10-1.98)	6.60	.01

Abbreviations: DRRI, Deployment Risk and Resilience Inventory; LF:HF, low-frequency to high-frequency ratio; MRS, Marine Resiliency Study; OR, odds ratio; PTSD, posttraumatic stress disorder.

[a] The MRS-I cohort included 1356 participants without PTSD before and after deployment and 59 individuals with PTSD after deployment.

[b] The MRS-II cohort included 720 participants without PTSD before and after deployment and 25 individuals with PTSD after deployment.

[c] The LF:HF ratio was log transformed.

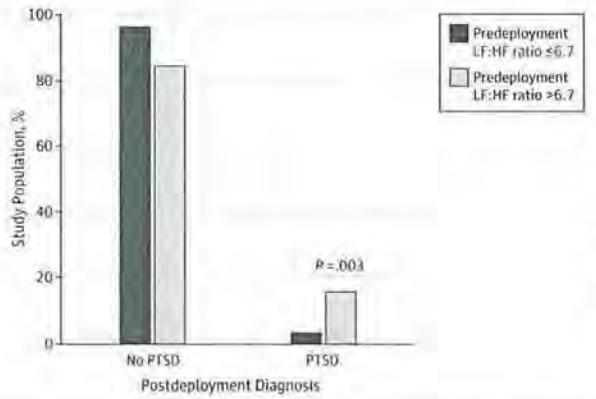
had a higher prevalence of PTSD after deployment than did participants with low predeployment LF:HF ratios. Figure 1 presents the flow of participants in both cohorts. Of the 1415 participants in MRS-I, 1356 individuals (95.8%) who had valid HRV data at the predeployment visit and also attended the 6-month postdeployment visit did not meet PTSD criteria at either visit, and 59 participants did not meet criteria for PTSD before deployment but met the criteria after being deployed (4.2% prevalence of postdeployment PTSD). In the MRS-II cohort of 745 participants, 720 individuals (96.6%) did not meet PTSD criteria at either visit, and 25 participants met the criteria for PTSD after deployment (3.4% prevalence of postdeployment PTSD). Demographic, military service, and clinical data comparing MRS-I and MRS-II are found in Table 1. Compared with MRS-I participants, those of the MRS-II cohort were younger, with lower predeployment CAPS scores, a higher prevalence of prior deployment, more time elapsed since the use of nicotine and caffeine, and a higher self-report of deployment-related stress (higher DRRI scores).

In MRS-I participants, the log-transformed LF:HF ratio was significantly associated with membership in the PTSD group such that higher LF:HF ratios at the predeployment visit were associated with new PTSD cases after deployment (OR, 1.63; 95% CI, 1.14-2.34; P = .008) (Table 2) (analyses of shrinkage and

performance of the regression model are in the eMethods in the Supplement). Neither the log-transformed LF (OR, 1.28; 95% CI, 0.92-1.73; $P = .21$) nor HF (OR, 0.91; 95% CI, 0.68-1.15; $P = .54$) achieved statistical significance in the logistic regressions.

Regression with the log-transformed LF:HF ratio was repeated in MRS-II participants. In that analysis, the LF:HF ratio was not statistically significant (OR, 1.20; 95% CI, 0.72-2.03; $P = .50$) (Table 2). Power to detect a significant effect in the MRS-II cohort was then calculated. Parameters used were the observed OR (1.63) of the LF:HF ratio in MRS-I, observed mean (SD) of the LF:HF ratio in MRS-I (0.36 [0.77]), no correlation between the LF:HF ratio and other covariates, and a 5% probability of PTSD under the null hypothesis of no effect of the LF:HF ratio. Power calculations suggested that 964 participants would have been required in MRS-II to achieve 80% power to detect an effect of the magnitude observed in MRS-I at $P = .05$. However, a meta-analysis of the weighted β values for the LF:HF ratio regression from both the MRS-I (OR, 1.63; 95% CI, 1.14-2.34) and MRS-II (OR, 1.20; 95% CI, 0.72-2.03) samples indicated that the LF:HF ratio was a statistically significant predictor of PTSD (OR, 1.47; 95% CI, 1.10-1.98; $z = 2.57$; $P = .01$) (Table 2). Figure 2 illustrates that the prevalence of postdeployment PTSD was higher in MRS-I and MRS-II participants with high (>2 SDs above the mean) predeployment LF:HF ratios (15.8% [6 of 38 participants]) compared with participants who did not have high LF:HF ratios (3.7% [78 of 2122 participants]).

Figure 2. Percentage of Marine Resiliency Study Participants With Low (n=2122) vs High (n=38) Predeployment Low-Frequency to High-Frequency (LF:HF) Ratios and Postdeployment Posttraumatic Stress Disorder (PTSD)



The LF:HF ratios were back transformed from natural log transformations. Ratios greater than 6.7 represent values greater than 2 SDs from the grand mean. χ^2 analysis was used.

When all covariates were accounted for in the multivariate logistic regression analyses for MRS-I participants, the log-transformed LF:HF ratio retained statistical significance as a predictor of PTSD group (OR, 1.57; 95% CI, 1.04-2.37; $P = .03$). Parameter estimates of this regression are reported in Table 3.

The multivariate regression model with the log-transformed LF:HF ratio was repeated in MRS-II participants. The LF:HF ratio was not statistically significant, but the meta-analysis of weighted β values for the MRS-I and MRS-II groups indicated that the LF:HF ratio was a statistically significant predictor of PTSD development (OR, 1.42; 95% CI, 1.02-1.98; $z = 2.05$; $P = .04$) (Table 3). Additional exploratory multivariate logistic regressions with the LF:HF ratio and each covariate in predicting PTSD in MRS-I and MRS-II participants are presented in the eTable in the Supplement.

Although some correlations between predeployment log-transformed LF and HF and postdeployment CAPS scores reached or approached statistical significance owing to the large sample size, the variance in symptom severity predicted by HRV was low (LF correlations with total CAPS: Pearson $r = -0.06$, $P = .03$; CAPS Avoidance-Numbing: Pearson $r = -0.07$, $P = .02$; and CAPS Arousal: Pearson $r = -0.07$, $P = .02$; HF correlations with CAPS Avoidance-Numbing: Pearson $r = -0.08$, $P = .01$ and CAPS Arousal: Pearson $r = -0.06$, $P = .04$). Post hoc correlations between HRV and other variables are presented in the eMaterial in the Supplement.

Table 3. Parameter Estimates for Multivariate Logistic Regressions Including the LF:HF Ratio and Additional Covariates in Predicting PTSD After Deployment

	MRS-I[a]			MRS-II[b]			Meta-analysis		
Characteristics	OR (95% CI)	Wald χ^2	P Value	OR (95% CI)	Wald χ^2	P Value	OR (95% CI)	Wald χ^2	P Value
Deployment-related TBI	2.92 (1.42-6.03)	8.47	.004	4.67 (1.92-11.40)	11.48	.001	3.52 (2.01-6.17)	19.30	<.001
DRRI after deployment	2.37 (1.41-3.99)	10.53	.001	1.82 (0.99-3.32)	3.77	.05	2.12 (1.43-3.14)	13.87	.001
LF:HF ratio[c]	1.57 (1.04-2.37)	4.52	.03	1.18 (0.67-2.06)	0.33	.57	1.42 (1.02-1.98)	4.21	.04
Predeployment CAPS score	1.04 (1.02-1.06)	15.01	<.001	1.05 (1.02-1.08)	9.49	.002	1.05 (1.03-1.06)	24.40	<.001
Ancestry[d]	NA	2.16	.54		1.67	.64	NA	NA	NA
Battalion[d]	NA	2.04	.56	0.80 (0.32-1.99)	0.23	.63	NA	NA	NA
Age	0.95 (0.43-1.07)	0.63	.43	0.92 (0.76-1.13)	0.63	.43	0.94 (0.85-1.05)	1.18	.28

Abbreviations: CAPS, Clinician-Administered PTSD Scale, DRRI, Deployment Risk and Resilience Inventory; LF:HF, low-frequency to high-frequency ratio; MRS, Marine Resiliency Study; NA, not applicable; OR, odds ratio; PTSD, posttraumatic stress disorder; TBI, traumatic brain injury.

[a] The MRS-I cohort included 1237 participants without PTSD before and after deployment and 44 individuals with PTSD after deployment. Some MRS-I participants were missing a full set of data for all covariates.

[b] The MRS-II cohort included 720 participants without PTSD before and after deployment and 25 individuals with PTSD after deployment.

[c] The LF:HF ratio was log transformed.

[d] These variables included multiple levels (except for the MRS-II battalion, with 2 levels), and the significance levels refer to an overall effect.

Discussion

Previous cross-sectional studies[11-13,19,31] repeatedly showed that lower HRV, thought to reflect inflexibility in the ANS response, is associated with PTSD. To our knowledge, the present study is the first large-scale report of a modest association between HRV before a potentially traumatic circumstance (in this case, combat exposure) and subsequent development of PTSD. The association was not observed when examining HRV variables that putatively isolate sympathetic and parasympathetic components, in contrast to previous findings of an association between lower predeployment HF and predeployment PTSD in this MRS sample.[19] Although those results suggested that, in this cohort of Marines, existing or chronic PTSD was most strongly associated with reduced parasympathetic activity, our present findings imply a role for pretrauma sympathetic activation (relative to parasympathetic activity) in influencing future vulnerability to significant trauma symptoms after combat exposure-related deployment. We did not observe meaningful correlations between HRV measures and symptom severity. Heart rate variability may not necessarily be associated with incremental changes in the severity of PTSD symptoms but may be a factor in or a harbinger for the development of the full syndrome and its associated adverse functional consequences.

The origin of the lower HRV observed in participants who eventually developed PTSD is unknown. A recent twin study[13] suggested that lower HRV is not found in the unaffected twin of veterans with PTSD and that low HRV is normalized with remission of PTSD symptoms. Taken together with the present results, low HRV may reflect an at-risk state rather than a trait. Low HRV may reflect the effects of environmental factors, perhaps recent, that increase PTSD risk. For example, a heightened stress response may contribute to the low HRV observed in the group that eventually developed PTSD since sensitivity to anxiety has been suggested as a PTSD vulnerability factor.[33] Deployment history was similar across groups, and this factor has not been strongly associated with HRV in past studies.[13] Nevertheless, it is possible that the PTSD risk group was exposed to more intense combat in previous deployments, or they may have experienced other adverse events that rendered them at greater risk for eventual development of PTSD. The results suggest that exposure to a combat-related deployment may not substantially affect ANS function for all military personnel; rather, there may be individuals who are particularly vulnerable to the serious psychological consequences of trauma. Resilience and vulnerability to PTSD are complex and multifactorial phenomena[34] that include genetic inheritance risk factors,[35,36] preexisting cognitive and psychological features,[37] lifetime trauma exposure especially early in childhood,[38] and perhaps also perturbations in ANS regulation. A recent review[16] posits that lower HRV may constitute a vulnerability factor for development of PTSD, perhaps because disrupted ANS function reflects perturbed cognitive and inhibitory control of stress response systems. Disruption in the neuroendocrine system that governs the stress response, the hypothalamic-pituitary-adrenal axis, may influence trauma vulnerability. The ANS is thought to play a role in the regulation of stress responses via inhibition of the hypothalamic-pituitary-adrenal axis by the vagus.[39] Thus, relatively increased sympathetic activation may reflect insufficient inhibition of the hypothalamic-pituitary-adrenal axis, leading to dysregulation of stress hormones and disruption of a normal stress response, which may ultimately contribute to vulnerability to PTSD following a traumatic event. Assuming that low HRV is associated with core mechanisms of PTSD risk and is not an epiphenomenon, an intriguing issue is whether PTSD risk can be decreased via methods intended to improve ANS function, such as biofeedback[40] and other interventions.[41,42] For example, there is a promising role for mindfulness-based interventions, particularly meditation, in increasing HRV.[43-45]

The present study's restriction to a young male group of US military personnel limits its generalizability to other PTSD populations. As our group[19] has previously suggested, the association between ANS function and trauma symptoms probably depends on the population and context of the traumatic event. Furthermore, the LF:HF ratio, although widely used, has been criticized for not always reflecting a robust and specific measure of sympathetic to parasympathetic balance,[46] particularly in situations when respiration is not accounted for, as in the present study (eg, respiratory sinus arrhythmia[47,48]). The assessment of many Marines in short time frames rendered a brief PPG recording to be the most practical method compared with longer recordings using electrocardiographic Holter monitors plus respiratory bands. Thus, we could not assess to what extent breathing rates may have moderated HRV. No significant association was observed between postdeployment PTSD and other predeployment HRV indices (ie, the LF and HF components); a small sample size for the PTSD group certainly may have reduced the power to detect these potential associations. The MRS-I and MRS-II cohorts were somewhat heterogeneous (Table 1 and Figure 1), which is why we conducted a meta-analysis instead of simply combining the 2 cohorts. Although the LF:HF ratio showed a similar effect size in MRS-II, it did not reach statistical significance in that cohort, likely owing to low power. Finally, in contrast to studies using 24-hour Holter monitoring recordings,[11,13] we did not observe a linear relationship between predeployment HRV and postdeployment PTSD symptoms. Previous studies[11,13] demonstrated cross-sectional—not longitudinal—correlations between HRV indices and PTSD symptoms. In the present study, a 5-minute HRV sample may have been insufficient to adequately capture an association between this relatively nonspecific physiologic measure and PTSD symptoms obtained more than 1 year later. Integrated analyses of HRV with other risk factors in this cohort (eg, markers of inflammation, fear processing, and traumatic brain injury) will help to elucidate the relative usefulness of this marker for predicting PTSD risk in active-duty military members.[18,20,32,49]

Conclusions

This investigation provides initial longitudinal evidence that ANS function may contribute to vulnerability and resilience

to PTSD along with known risk factors, such as combat exposure and preexisting stress and trauma symptoms. If supported, this association sheds additional light on the interplay between complex biological systems and the psychological and functional consequences of trauma and may provide new opportunities for prevention.

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Study concept and design: Minassian, Baker, Nievergelt, Geyer, Risbrough.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Minassian, Baker, Nievergelt.
Critical revision of the manuscript for important intellectual content: All authors.
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Review Article

The Psychiatric Genomics Consortium Posttraumatic Stress Disorder Workgroup: Posttraumatic Stress Disorder Enters the Age of Large-Scale Genomic Collaboration

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The development of posttraumatic stress disorder (PTSD) is influenced by genetic factors. Although there have been some replicated candidates, the identification of risk variants for PTSD has lagged behind genetic research of other psychiatric disorders such as schizophrenia, autism, and bipolar disorder. Psychiatric genetics has moved beyond examination of specific candidate genes in favor of the genome-wide association study (GWAS) strategy of very large numbers of samples, which allows for the discovery of previously unsuspected genes and molecular pathways. The successes of genetic studies of schizophrenia and bipolar disorder have been aided by the formation of a large-scale GWAS consortium: the Psychiatric Genomics Consortium (PGC). In contrast, only a handful of GWAS of PTSD have appeared in the literature to date. Here we describe the formation of a group dedicated to large-scale study of PTSD genetics: the PGC-PTSD. The PGC-PTSD faces challenges related to the contingency on trauma exposure and the large degree of ancestral genetic diversity within and across participating studies. Using the PGC analysis pipeline supplemented by analyses tailored to address these challenges, we anticipate that our first large-scale GWAS of PTSD will comprise over 10 000 cases and 30 000 trauma-exposed controls. Following in the footsteps of our PGC forerunners, this collaboration—of a scope that is unprecedented in the field of traumatic stress—will lead the search for replicable genetic associations and new insights into the biological underpinnings of PTSD.
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INTRODUCTION

Posttraumatic stress disorder (PTSD) occurs in only a minority of persons exposed to traumatic events (Breslau *et al*, 1998; Kessler *et al*, 1995). Factors that influence PTSD susceptibility include sex, age, early life adversity, the nature,

and timing of traumatic event exposure(s), the cumulative burden of these exposures, as well as various other psychosocial and personality factors (Zoladz and Diamond, 2013). In the US, race/ethnicity impacts the rate, type, and age at traumatic-event exposure, as well as the risk for development of PTSD after exposure (Roberts *et al*, 2011). Moreover, some events are more pathogenic than others. Events of an interpersonal nature, eg, rape, partner violence, and assault, confer greater risk of developing PTSD than other types of trauma, eg, natural disasters (Kessler *et al*, 1995). Twin studies have indicated that risk of exposure to some types of trauma may itself be heritable, which is known as a gene–environment correlation (rGE) effect. Lyons *et al.* (1993) using data from the Vietnam Era Twin Registry (Eisen *et al*, 1987; Goldberg *et al*, 1987), found that the heritability of combat exposure ranged from 35 to 47%. In civilians, Stein *et al.* (2002) found that exposure to interpersonal traumatic events was modestly heritable (~20%). The rGE for trauma exposure appears to be largely explained by genetic influences on personality (Afifi *et al*, 2010; Jang *et al*, 2003). For example, sensation seeking is a heritable personality trait that is characterized by engaging in behavior, such as driving at high speeds (Zuckerman, 1994), which may increase the likelihood of trauma exposure. In addition, the risk of PTSD following trauma exposure is moderately heritable, even after controlling for the genetic influences on trauma exposure. Twin studies established that genetic influences explain a substantial proportion of vulnerability to PTSD, from ~30% in male Vietnam veterans (True *et al*, 1993), to 38% in a sample of male and female civilians (Stein *et al*, 2002), with an upward heritability estimate of 72% in young women (Sartor *et al*, 2011). This is comparable to other internalizing disorders such as major depressive disorder and panic disorder (Kendler and Prescott, 2007). Furthermore, genetic influences on PTSD may overlap with those for other mental disorders. The genetic influences on major depressive disorder and PTSD may substantially overlap (Fu *et al*, 2007; Koenen *et al*, 2007; Sartor *et al*, 2012). Phenotypes like alcohol and drug dependence (Sartor *et al*, 2011; Xian *et al*, 2000) and nicotine dependence (Koenen *et al*, 2005) share ~40% genetic risk with PTSD. Genetic influences common to generalized anxiety disorder and panic disorder symptoms account for ~60% of the genetic variance in PTSD (Chantarujikapong *et al*, 2001).

The search for genetic markers of PTSD is a relatively new endeavor, with the majority of studies conducted within the last decade. These investigations involve genotyping (measuring variation at) a particular location along the genome. Individuals' particular genetic code (genotype), at a location (locus) is then compared for a sample of cases and controls. Most research to date has employed the candidate-gene approach, in which genes are selected for study based on their theorized involvement in biological pathways implicated in the pathophysiology of PTSD. Given that PTSD has historically been conceptualized as a disorder of pathological fear and stress (Wilker and Kolassa, 2013), most studies have focused on candidate genes involved in biological systems associated with the fear response, including the hypothalamic–pituitary–adrenal axis (eg, *FKBP5*, *CRHR1*) and the locus coeruleus–noradrenergic system (eg, *COMT*, *ADRB1*, and *ADRB2*). Additional work has examined serotonergic and dopaminergic systems involved in the neural pathways underlying emotion (eg, *SLC6A4*, *SLC6A3*), and systems involved in memory consolidation and stabilization (eg, *WWC1*, *PRKCA*). Candidate gene studies of PTSD have produced a large body of literature (Pitman *et al*, 2012; Wilker and Kolassa, 2013). However, candidate gene studies have, for the most part, failed to replicate when the definition of replication is restricted to the observation of a significant association in the same allele with the same effect direction (see Sullivan (2007) for a discussion of replication in candidate genes studies). To date, relatively few candidate gene studies of PTSD have examined gene–environment (GxE) interactions, an approach that may be particularly well-suited for examining genetic risk in PTSD. However, candidate gene GxE studies in psychiatric literature have been prone to false positives and suitable replication has been difficult to obtain (Duncan and Keller, 2011). Thus, as in the larger psychiatric genetics literature (Psychiatric Gwas Consortium Coordinating Committee *et al*, 2009), for the most part, robust support for markers associated with risk or resilience for PTSD has not emerged from candidate gene studies.

In contrast to candidate gene studies, in a genome-wide association study (GWAS), genetic variation—primarily single-nucleotide polymorphisms (SNPs)—is examined without hypothesizing the role of any particular gene or biological function (Psychiatric Gwas Consortium Coordinating Committee *et al*, 2009). The viability of a GWAS strategy is predicated on the relatively low cost of chip-based genotyping that reliably and cheaply assesses thousands or even millions of SNPs distributed throughout the genome. Chip-based genotyping cannot yield information about rare or even private (present in only one person or shared within a particular family) mutations, except in the case of rare or private large copy number variants (CNVs) that can be detected by examining the assays across multiple SNPs. To examine other types of rare variants, more costly whole-genome or whole-exome sequencing is required. Consequently, the investigation of rare variants has primarily been addressed through sequencing, whereas common-variant associations have been assessed through chip-based genotyping. It is customary to examine hundreds of thousands or millions of SNPs in a single GWAS. As the number of SNPs examined is great, and the number with individually detectable effects is presumably small, strict multiple-testing control is required to reduce the number of false positives. The current customary significance threshold is $P<5\times10^{-8}$ for a genome-wide study regardless of the particular number of SNPs examined. This strict threshold is useful in that it gives some assurance that the detected loci will be robustly associated with the disorder under study.

To date, four GWAS of PTSD have been published (Guffanti *et al*, 2013; Logue *et al*, 2013; Nievergelt *et al*, 2015; Xie *et al*, 2013). The genome-wide significant findings of each are summarized in Table 1. Although the roles of these GWAS-identified genes in risk for PTSD have not been elucidated, the top loci identified in the extant GWAS have been implicated in a variety of processes, including neuroprotection, actin polymerization, neuronal function, and immune function (Almli *et al*, 2014b; Guffanti *et al*, 2013; Logue *et al*, 2013; Xie *et al*, 2013). Notably, the GWAS have identified variants in novel pathways that would not

Table 1 Results of GWAS of PTSD

First author (year)	Number of markers	Discovery sample(s)		Sample	Gender	Ancestry	Replication sample(s)		Characteristics	Locus	Genes (SNP)	p-value
		Number of cases	Number of controls				Number of cases	Number of controls				
Logue et al (2013)	2.5 million	295	196	Trauma-exposed veterans and their intimate partners	60% male	EA	(a) 43	41	AA trauma-exposed veterans and their intimate partners	15q22.2	*AQRA* (rs80421493)	2.5×10^{-8}
							(b) 100	421	AA trauma-exposed community sample			
Xie et al (2013)	~870 000	(a) 300	1278	Participants in genetics of substance use studies; trauma-exposed controls only in secondary analysis	60% male	EA	(a) 207	1692	EA participants in genetics of substance use studies	7p12. 4q32.	*COBL* (rs4906001) *TLL1* (rs6812849)	3.97×10^{-9} 2.99×10^{-7}
		(b) 444	2322	Participants in genetics of substance use studies; trauma-exposed controls only in secondary analysis	55% male	AA	(b) 89	655	AA participants in genetics of substance use studies	—	—	—
Guffanti et al (2013)	730 525	94	319	Trauma-exposed community sample	100% female	83% AA	578	1963	EA trauma-exposed female nurses	2q32.1	Long intergenic non-coding RNA *AC006518.1* (rs10170218)	5.09×10^{-8}
Nievergelt et al (2015)	888 113 directly genotyped; >21 million imputed	940	2564	Trauma-exposed military sample	100% male	86% EA, 25% Hispanic[a]	313	178	EA trauma-exposed veterans and their intimate partners	10p12.1	*PRTFDC1* (rs6482463)	2.04×10^{-9}

Abbreviations: AA, African American ancestry; EA, European American ancestry; GWAS, genome-wide association study; PTSD, posttraumatic stress disorder; SNP, single-nucleotide polymorphism.
[a]Conducted analyses separate.

have been examined using the biologically driven candidate-gene methodology. So far, the findings from the different studies have not consistently implicated a primary set of PTSD risk loci. Numerous factors may contribute to this, including one or more of them being false positives, heterogeneity across samples, and a statistical artifact of the 'winner's curse' which implies that effect size estimates will be inflated for moderately powered studies (Xiao and Boehnke, 2009). It is important to note that samples sizes under 5000 or even 10 000 are now considered to be relatively 'small' by modern GWAS standards (Sullivan *et al*, 2012). Convincing demonstrations of association now come from GWAS of tens or even hundreds of thousands of individuals (Lango Allen *et al*, 2010).

THE PGC AND PROGRESS IN PSYCHIATRIC GENETICS

Although the results of the PTSD GWAS published to date may prove useful, experience from GWAS of other psychiatric disorders has made it clear that large-scale collaborations are necessary to yield consistently replicable findings. The Psychiatric Genomic Consortium (PGC) was organized in 2007 as an outgrowth of the Genetic Association Information Network—a joint public–private funded venture to study attention deficit/hyperactivity disorder (ADHD), diabetic nephropathy, major depressive disorder, psoriasis, schizophrenia, and bipolar disorder (Gain Collaborative Research Group *et al*, 2007). The PGC had as its goal to conduct GWAS studies of ADHD, bipolar disorder, major depressive disorder, and schizophrenia, and later autism spectrum disorder (Psychiatric GWAS Consortium Coordinating Committee *et al*, 2009; The Psychiatric GWAS Consortium Steering Committee, 2009). The PGC was designed to bring together psychiatric GWAS from around the world to enable adequately powered analyses. By centralizing analyses under a consortium umbrella, the PGC has overcome the substantial challenges of harmonizing quality control procedures, analytic methods, and phenotype definitions to enable GWAS meta- and mega-analyses (Sullivan, 2010). By adequately powering analyses, and standing by strict definitions of significance from the outset, the PGC has encouraged the production of high-quality replicable genetic associations.

The PGC has become the largest collaborative effort in the history of psychiatry and, as of this writing, comprises more than 500 scientists from more than 100 countries. More than 172 000 subjects have been included, and genotyping of an additional 90 000 is currently underway. PGC efforts have established that sufficiently powered GWAS is a viable strategy for identifying neuropsychiatric disorder susceptibility loci for bipolar disorder (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011) and schizophrenia (Ripke *et al*, 2011). The PGC has enabled discovery of a large number of reliably associated genetic loci, 108 for schizophrenia alone at last count (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). The PGC analyses have also given us an insight into the genetic architecture of psychiatric disorders (Collins and Sullivan, 2013). In particular, these analyses have demonstrated that psychiatric disorders are polygenic (having hundreds or even thousands of risk loci) and that common variation accounts

for a substantial component of the underlying genetic architecture. Their results have indicated that GWAS-significant loci represent the tip of the iceberg in terms of the proportion of variance explained by inherited genetic variation, and the remaining variation is likely to represent a mix of rare and common genetic effects. For example, in schizophrenia, the proportion of variation explained by the 108 genome-wide significant loci was 3.4% (Schizophrenia

Table 2 Summary of Participating PGC–PTSD Groups

Principal investigator	Sample name	Cases	Controls	Total	Ancestry	Illumina platform
Previously genotyped samples						
Ressler, Kerry	Grady trauma project	1503	3249	4752	AA—Mixed	1M Omni-Quad
Aiello, Allison	Detroit Neighborhood Health Study	192	620	812	AA	OmniExpress
Gelernter, Joel	Genetics of substance dependence	818	4633	5451	60% EA	OmniQuad
Nievergelt, C.	Marine Resilience Study	538	3477	4015	EA 60%	OmniExpressExome
Bierut, Laura	Family Study of Cocaine Dependent	471	3568	4039	Mixed	1M Beadchip
Miller, Mark	Boston-VA	600	500	1100	Mixed	Omni 2.5M
Stein, Murray	Army STARRS	4500	15 500	20 000	Mixed	OmniExpressExomeC
Beckham, Jean	MIRECC	1156	1156	2312	Mixed	650/1M-Duo/Omni2.5
Ressler, Kerry	Grady trauma project	497	1751	2248	AA—Mixed	1M Omni-Quad
Stein, Murray	VA Cooperative Study	10 000	10 000	20 000	Mixed	OmniExpressExomeC
DeLisi, Lynn	UCSD VA	1000	1000	2000	Mixed	
Smith, Nicholas	VET Study	492	377	869	Mixed	
Hollegaard, Mads	Danish Blood Spot Cohort	500	2500	3000	EA	
	Subtotal	22 267	48 331	70 650		
Samples with funded genotyping						
Koenen, Karestan	Nurses Health Study II	680	700	1380	EA	PsychChip
Liberzon, Israel	Ohio national Guard Study	170	200	370	EA	PsychChip
Lyons, Michael	Vietnam Era Twin Registry	350	350	700	EA	PsychChip
Ressler, Kerry / Dan Stein	Civilian South African Cohort	200	400	600	S. African	PsychChip
Vermetten, Eric	Military Research (PRISMO)	35	965	1000	EA	PsychChip
Bryant, Richard	Australian Injury Vulnerability Study	205	796	1001	EA	PsychChip
Ressler, Kerry	Predictive Biomarkers Project	200	400	600	80% AA	PsychChip
	Subtotal	1840	3811	5651		
Additional samples identified for future genotyping once funding is obtained						
Ressler, Kerry	Grady Trauma Project*	200	1000	1200	AA—mixed	
Ressler, Kerry / Holly Orcutt	NIU Shooting Sample	70	230	300	80% EA	
Aiello, Allison	Detroit Neighborhood Health Study	72	197	269	AA	
Liberzon, Israel	Gracy Detroit Mother's Study	200	220	420	75% EA, 23%AA	
Liberzon, Israel	Ohio national Guard Study	10	860	870	85% EA, 13%AA	
Koenen, Karestan	Nurses Health Study II	170	1463	1633	EA	
Kessler, Ronald	World Mental Health Surveys	318	6969	7287	Other	
Amstadter, Ananda	Service Experience and Alcohol Preference Study	80	80	160	80% EA, 20%AA	
Amstadter, Ananda	Disaster-affected adolescents and families	82	698	780	70% EA, 25% AA	
Yehuda, Rachel	Improving PTSD outcomes in OIF/OEF returnees	121	300	421	Mixed	
Yehuda, Rachel	Suicidality and PTSD	90	0	90	Mixed	
Yehuda, Rachel	Holocaust PTSD	45	0	45	EA	
Feder, Adriana	World Trade Center responders	50	200	250	Mixed	
Baker, Dewleen	Marine Resiliency Study*	117	583	700	EA 60%	
Stein, Murray	Army STARRS	1800	12 200	14 000		
Bradley, Bekh	Genetic and Environmental Risk/Resilience Factors for Posttraumatic Stress Disorder in OEF/OIF Veterans	200	600	800	AFR	
Beckham, Jean	MIRECC*	152	758	910	Mixed	
Herringa, Ryan	Neural Basis of Emotion Regulation in PTSD	50	50	100		
Bisson, Jonathan	Wales PTSD Study	462	960	1422	EA	
Hollegaard, Mads	Danish Blood Spot Cohort	20 000	20 000	40 000	EA	
Risbrough, Victoria	Norman VA exposure therapy	200	0	200	60% EA	
	Subtotal	25 489	51 368	76 857		
	TOTAL	48 596	99 510	148 158		

Abbreviations: AA, African American ancestry; EA, European American ancestry; PGC, Psychiatric Genomics Consortium; PTSD, posttraumatic stress disorder.
*Study contributing more than one wave of data.

Working Group of the Psychiatric Genomics Consortium 2014), whereas estimates of the total proportion of variation explained by common genotyped SNPs has been estimated to be approximately 25 and 45% depending on the population and method used (International Schizophrenia Consortium *et al*, 2009; Lee *et al*, 2012; Ripke *et al*, 2013). In addition to common variants, rare variants such as CNVs were found to explain a proportion of risk for schizophrenia, bipolar disorder, and autism (Malhotra and Sebat, 2012). Results in schizophrenia also suggest that many of the genome-wide significant loci obtained at smaller sample sizes will turn out to be significant once the sample size gets large (Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). The polygenic nature of the psychiatric disorders is such that once the sample size is sufficiently large, the genome-wide distribution of association statistics will differ from the expected null distribution (Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). A new method called LD regression has been developed to test whether or not genomic inflation in this context represents a polygenic risk component to disease or inflated significance due to the population substructure (Bulik-Sullivan *et al*, 2015b).

Also importantly, as the list of risk loci has expanded, they have begun to coalesce into biological pathways, illuminating disease pathogenesis and implicating new targets for drug development (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Nurnberger *et al*, 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). For example, recent analyses have highlighted the role of immune system and glutamatergic function in schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014) and calcium channel signaling across childhood- and adult-onset disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). The PGC Cross-Disorder Workgroup identified several loci that appear to confer risk across autism, ADHD, bipolar disorder, major depressive disorder, and schizophrenia (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). Aggregate genome-wide analyses (using SNP-heritability estimates and polygene scores) showed significant genetic overlap among these disorders, with the strongest overlap between bipolar disorder and schizophrenia (genetic correlation = +0.68; Cross-Disorder Group of the Psychiatric Genomics Consortium *et al*, 2013). By highlighting shared biologic vulnerability, this work may inform efforts to refine psychiatric nosology. Recently Bulik-Sullivan *et al* (2015a) have developed a new computationally efficient technique for estimating cross-trait genetic correlation based on LD regression. The results obtained with this new method mirror earlier work showing genetic correlation between schizophrenia and bipolar disorder. However, this new method has the advantage that it can be run on summary statistics from both traits, rather than necessitating individual-level data.

The PGC has also led the development of the PsychChip. The PsychChip is an Illumina (San Diego, CA) genotyping chip that assesses ~560 000 markers. It is designed to be suitable for analysis of psychiatric traits and for use in the imputation of genome-wide SNP genotypes (described in the Supplementary Materials).

THE PGC-PTSD WORKGROUP

Drs Koenen, Ressler, and Liberzon founded the PCG-PTSD Workgroup (PGC-PTSD) in May 2013 with a satellite meeting at the Society of Biological Psychiatry co-sponsored by NIMH and One Mind, a patient advocacy non-profit organization (http://onemind.org). The PGC-PTSD has, as its goal, the bringing together of a large number of PTSD researchers for the purpose of large-scale GWAS studies of PTSD.

The Sample

The size and characteristics of the groups anticipated to participate in the PGC-PTSD are summarized in Table 2. First, six groups have uploaded genotype data that will be used in the initial PGC-PTSD analysis. This includes a combined sample size of 20 468 subjects (4487 cases and 15 981 controls). Second, an additional 53 552 subjects from 13 studies have been genotyped (19 408 cases and 34 144 controls). Third, there are 20 studies with genotyping in process or planned ($N = 71\,757$; 24 439 cases and 47 318 controls). Many of these studies will be using the PsychChip. Data collection sites are from across the US (eg, Atlanta, San Diego, New Haven, Detroit) and include three additional countries (Denmark, The Netherlands and South Africa). Like other PGC disorders, we expect that this initial sample is merely the first foray into large-scale meta-analyses.

The vast majority (>80%) of controls across these studies have experienced a trauma fulfilling the exposure criterion for PTSD. Hence, the PGC-PTSD sample will have a large trauma-exposed control group available for comparison with PTSD cases. Focusing on trauma-exposed individuals may be useful, as any PTSD risk allele, which will have an increased rate in PTSD cases, will presumably have a lower frequency in PTSD-negative trauma-exposed controls than in trauma negative or unscreened controls. Hence, all other things being equal, the greater difference in allele frequency between PTSD cases and trauma-exposed controls will lead to a greater power to detect the associations than a sample that includes trauma-negative or unscreened controls. Utilizing unscreened controls in the presence of rGE effects could result in associations representing a mix of loci, some of which were associated with risk of PTSD in the presence of trauma exposure and some of which were related to the risk of trauma exposure itself. The use of only trauma-exposed controls and inclusion of degree of trauma exposure as a covariate in analyses should be adequate to place our focus tightly on loci that increase risk of PTSD directly.

Phenotype and Exposure Measurement Complexity

The harmonization of data across PGC–PTSD groups, like that for other psychiatric disorders, is complicated by variability in the assessment methods used. Two major approaches to the assessment of PTSD symptoms and diagnosis—structured clinical interviews and self-report instruments—are represented, with the primary distinction between them related to the source of the data (ie, clinician ratings *vs* participant self-report). Of the six samples already uploaded to the PGC–PTSD, five used self-report measures and one used clinician ratings. The major limitation of

clinical interviews is the considerable time and expense involved in training and administration, which renders this approach impractical for many studies. Studies featuring some of the largest samples have used self-report instruments to assess symptoms and estimate diagnosis. Additionally, although not yet investigated within the PGC–PTSD group, there is the possibility of using diagnostic information from additional sources such as from electronic health records, which can provide evidence of convergent validity. Finally, methods for determining diagnostic status (ie, cases *vs* controls) differ between interview and self-report approaches, as well as across traumatized populations. Interview-based studies, based on the judgment of trained clinicians, typically apply the DSM algorithm (ie, for DSM-IV, one reexperiencing symptom, three avoidance and numbing symptoms, and two hyperarousal symptoms). With self-report measures, diagnostic classifications are somewhat less straightforward. The DSM-IV algorithm can also be applied by defining symptoms endorsed above a given severity threshold level as present (ie, causing moderate or greater distress). However, patterns of item endorsement tend to vary across items and populations, so the application of a uniform criterion can yield significant differences in composition of cases across samples. Alternatively, PTSD diagnostic status can be determined in relation to a total symptom severity score cutoff. Studies that have examined the relationship between probable diagnoses derived from this approach *vs* interview measures of PTSD have found acceptable, though not excellent, agreement (see eg, McDonald *et al*, 2009; McDonald and Calhoun, 2010). Studies have shown that for any given measure the optimal score for differentiating cases from controls differs across samples and can be influenced by a host of factors, most notably, the base rate of the diagnosis in the sample (for a meta-analysis of PTSD Checklist (PCL) studies, see Terhakopian *et al*, 2008). Thus, because the same instrument can yield different classification performance across different samples, our cutoff score selections will take into account independent estimates of the true base rate of the sample.

The harmonization of measures of trauma exposure across studies is an additional complication for PTSD genetics research. Though the DSM offers a broad definition of the types of events known to cause PTSD, there is no uniform or generally agreed-upon framework for categorizing or measuring them. A variety of self-report measures of trauma exposure are represented among PGC–PTSD studies. Most consist of a list of events that meet the DSM-IV PTSD Criterion A1 trauma definition including exposure to sexual or physical assault, combat or warfare, sudden death of friend/loved one, and so on. Most also make it possible to reclassify events for harmonization purposes into broader categories such as childhood *vs* adult trauma, or interpersonal versus non-interpersonal trauma, or to compute a measure of total trauma load (ie, a sum of event exposure categories across the lifespan).

The instruments used in the various studies also differ with respect to the way that they link PTSD to the trauma. In clinical interview instruments such as the Clinician Administered PTSD Scale for DSM-IV (Blake *et al*, 1990) and the Structured Clinical Interview for DSM-IV Axis I Disorders (First *et al*, 1994), interviewers identify an index event(s) and then evaluate its link to subsequent symptoms while accounting for confounding factors such as comorbidity, substance abuse, medical issues, and reporting style. For self-report measures (eg, the PCL; Weathers *et al*, 1993), approaches range from those that link symptoms to a single event, to those that do not reference a single event, to those that reference military experience broadly.

Ancestry

Most extant PGC GWAS have been restricted to a single ancestral population because population stratification can lead to Type I and Type II errors in genetic association studies (Marchini *et al*, 2004). Psychiatric research in the US and Europe has traditionally enrolled a relatively large proportion of subjects of European ancestry, and consequently, GWAS in the PGC have been performed primarily using subjects of European ancestry (Figure 1a). In contrast, PTSD studies have recruited subjects primarily from high-risk populations, such as combat-veteran cohorts, or in urban areas with high rates of violent crime, and thus PGC–PTSD samples include a large proportion of subjects of African-American and Hispanic/Latino ancestry (Figure 1b). GWAS on such heterogeneous and admixed samples require additional considerations (eg, a study by Pasaniuc *et al* (2011)). Combining across multiple ancestry groups via meta-analysis has become more common in the recent past (see eg, Nievergelt *et al*, 2015 and Li and Keating, 2014 for review).

RESEARCH STRATEGY

PTSD Meta-Analysis

Our proposed analysis strategy is described in the Supplementary Materials and is briefly outlined here. Standardized quality control procedures and GWAS analysis based on the PGC GWAS analysis pipeline will be used (Ripke *et al*, 2013). Harmonized versions of continuous predictive variables and outcomes (eg, PTSD severity) will be generated based on within-study normalization of the instruments available. Categorical outcome and predictor variables will, for the most part, be based on the diagnostic schema adopted by the principal investigator of the particular study taking into account sample and measurement factors that affect prevalence estimates. The efficacy of the harmonization will be assessed using the descriptive statistics and by examining correlations between predictive variables, outcomes, and reported demographic information from each group. Our primary analysis will be a GWAS meta-analysis of PTSD followed by a GWAS of PTSD severity, both controlling for potential sources of bias as well as trauma-exposure variables. Based on a consensus of participating group members at in-person PGC–PTSD planning meetings, we determined to utilize dichotomous DSM-IV diagnosis as the primary phenotype. Initially, this analysis will be restricted to trauma-exposed controls. The pipeline will be modified to account for greater population stratification between and within PGC–PTSD groups compared with the typical PGC analysis. Both within-ancestral group and cross-ancestral group meta-analysis will be

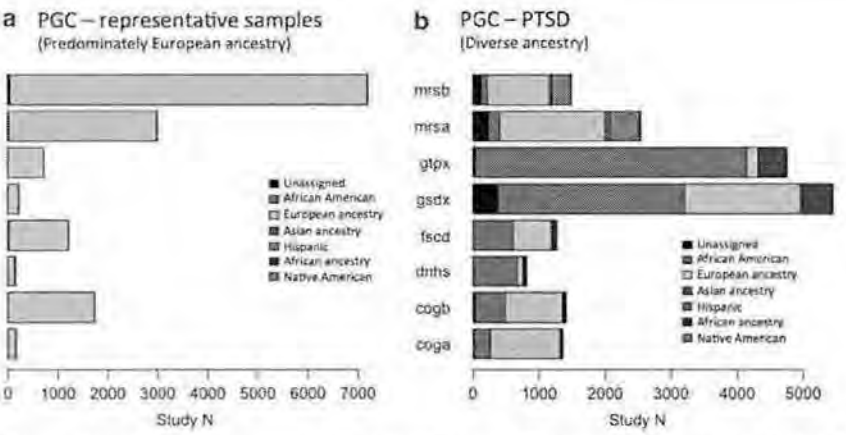

Figure 1 A comparison of ancestral diversity in (a) representative Psychiatric Genomics Consortium (PGC) samples of primarily European ancestry and (b) representative PGC–PTSD samples. Key: mrsa, mrsb—subsets (a and b) of the Marine Resilience Study (Nievergelt); gtpx—Grady Trauma Project (Ressler); gsdx—Genetics of Substance Dependence (Gelernter); fscd—Family Studies of Cocaine Dependence (Bierut); dnhs—Detroit Neighborhood Health Study (Aiello); cogb, coga—subsets (a and b) of the COGEND study (Bierut); Note that African American refers to subjects from the USA who typically have a mix of African and European ancestry, whereas African Ancestry refers to subjects from Africa without admixed ancestry. PTSD, posttraumatic stress disorder.

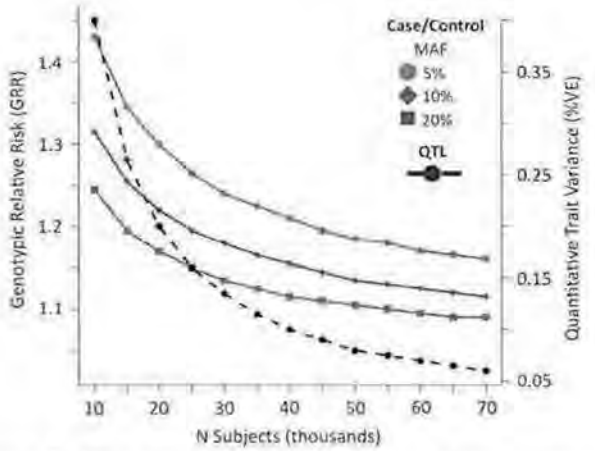

Figure 2 Effect size necessary to have 80% power for case-control and quantitative-trait association analyses demonstrating the relation between increasing sample size and ability to detect loci of smaller effect sizes. Key: calculated assuming PTSD prevalence of 15%, additive model, a type I error rate of 5×10^{-8}, and perfect LD between marker and trait allele for MAF > 5%). Calculations were based on a 1 : 3 PTSD case-control ratio or quantitative traits such as PTSD symptoms. PTSD, posttraumatic stress disorder.

performed. Subsequent investigation will include analyses of rare variants, including structural variants such as CNV.

The PGC–PTSD has already assembled a substantial aggregate sample size, as well as an extensive set of samples that will be genotyped if funding allows. The power to detect a SNP effect in a GWAS analysis varies as a function of the size of the effect, the allele frequency of the SNP, and the sample size. This is illustrated in Figure 2, which displays the minimum effect size that yields 80% power as a function of the SNP allele frequency and sample size. An analysis including the 45 000, the samples that have currently funded genotyping will have 80% power to detect a locus with a genotype relative risk (GRR) between 1.2 and 1.11 for allele frequencies between 5 and 20%. Increasing the sample size to 60 000 will allow us to detect a locus with a GRR between 1.17 and 1.1, respectively.

GxE Analyses

In addition to the standard GWAS meta-analysis, a secondary aim of the PGC–PTSD is to conduct a series of GxE analyses. Some readers may be surprised that this is not the primary analysis for PTSD. Although we are well aware of the conceptual relevance of GxE models to PTSD, the statistical challenges associated with GxE analyses are formidable. First, although PTSD clearly results from the interaction of trauma with genetic predisposition, it is unclear whether or not the biological realities of such an interaction are captured by testing deviations from a multiplicative logistic regression model (Thompson, 1991). Second, the significance of the GxE interaction term estimated using standard regression models can be inflated under commonly occurring conditions (Almli *et al*, 2014a; Voorman *et al*, 2011). Third, obtaining reasonable power in GxE analysis takes sample sizes larger than those required for main effect analyses. A sample four times as large has been proposed as a rule of thumb (Thomas, 2010). Finally, the power and bias of different GxE analysis methods vary depending on the nature of the interaction (Cornelis *et al*, 2012; Mukherjee *et al*, 2012).

Approaches used previously in PTSD genetics studies have ranged from including cumulative lifetime 'trauma load' as a covariate in the analysis (Kolassa *et al*, 2010) to explicitly testing for GxE interactions (Digangi *et al*, 2013). To date, there have been no genome-wide GxE studies of PTSD. Although the single genome-wide GxE study published in psychiatry to date (a study of ADHD) did not yield significant findings (Sonuga-Barke *et al*, 2008), genome-wide GxE studies have been successful in other areas (eg, Beaty *et al*, 2011).

The PGC–PTSD will use a two-stage strategy to examine GxE effects. First, given the likelihood of developing PTSD

increases with exposure to childhood trauma, interpersonal violence, and with increasing trauma load, GxE models will test the hypothesis that the effects of risk variants for PTSD (identified through the primary GWAS) are moderated by these environmental variables. The second approach is a 'genome-wide GxE' meta-analysis approach that will systematically interrogate the genome for GxE effects between SNPs and these three environmental variables. This will include fitting a logistic regression model of PTSD and linear model of PTSD severity as a function of a SNP × childhood trauma, SNP × interpersonal trauma, and SNP × total trauma load interaction effects using robust SEs to combat genome-wide inflation of significance. Follow-up analyses will examine the effect of multiple characteristics of trauma exposure, including trauma load, type, timing, and severity. Finally, we note that the data gathered here will provide a resource for secondary analysis and methodological development, as has been the case for other PGC disorders such as schizophrenia.

Comorbidity

PTSD is highly comorbid with other psychiatric disorders, and a substantial proportion of this comorbidity may be explained by common genetic influences (Koenen *et al*, 2003; Wolf *et al*, 2010). Hence, in this context comorbidity may present an opportunity to explore potential overlapping genetic effects in our sample. We propose to follow the PGC cross-disorder model and perform a polygenic architecture analysis with polygenic risk scores and LD regression to determine the proportion of genetic variance (heritability) common across PTSD and other psychiatric disorders.

PGC-PTSD SUBGROUPS

The PGC-PTSD also represents the confluence of vast reserves of PTSD-related information that will enable large-sample investigations of PTSD-associated epigenetic, neuroimaging, and other neurobiological measures. In order to facilitate the analysis of these data, a pair of focus groups have been created within the PGC-PTSD workgroup: the PGC-PTSD Epigenetics Workgroup and the PGC-PTSD Neuroimaging Genetics Workgroup.

PGC-PTSD Epigenetics Workgroup

Recently, 'stand alone' genome-scale studies of PTSD epigenetics and gene expression have provided initial insight into molecular dysregulation associated with the disorder (Mehta *et al*, 2013). Epigenetics provides a molecular context to GxE interactions by offering a biological mechanism through which gene expression can vary in response to an environmental exposure (see eg, Latham *et al*, 2012). Genetic variation has been shown to influence DNA methylation and gene expression levels, often in tissue-specific and developmental stage-specific manners; so-called methylation trait quantitative loci (meQTLs) and expression trait quantitative loci (eQTLs) have been identified across the genome in numerous studies to date (see eg, Smith *et al*, 2014). Although genome-scale studies of PTSD-associated meQTLs and eQTLs have yet to be reported, focused candidate gene studies have revealed notable examples of each (see eg, Klengel *et al*, 2013; Mehta *et al*, 2011; Ressler *et al*, 2011). Within the PGC-PTSD, there are several groups with both genome-wide genotype and methylation data, with a current total $n = 1114$. The PGC-PTSD Methylation Workgroup has, as its goal, the creation of a large PTSD-focused methylation data set that can be used to identify whether gene expression or methylation act as mediators of the association between SNPs and PTSD risk as well as identifying PTSD-relevant eQTLs and meQTLs that can be examined for association to PTSD and trauma exposure.

PGC-PTSD Neuroimaging Workgroup

The PGC group members have a large number of participating groups with neuroimaging data. Within the PGC-PTSD there are over 5000 samples that will have both structural MRI and GWAS data available. Smaller data sets of DTI, resting-state fMRI, MEG, and other imaging modalities are also available. These data will allow the investigation of how genomic markers modulate neuroimaging quantitative traits (QTs) associated with PTSD. The uncertainty associated with psychiatric nosology makes reference to an intermediate biological variable attractive, as the heritability of intermediate phenotypes such as regional brain volumes is often 80% or higher (den Braber *et al*, 2013). However, these will not represent a magic bullet. Given the results of the ENIGMA group, a neuroimaging GWAS meta-analysis consortium, effect sizes observed for individual SNPs on brain structures are likely to be modest and require large sample sizes to be adequately powered (Hibar *et al*, 2015; Stein *et al*, 2012). The PGC-PTSD Neuroimaging Workgroup will facilitate the creation of a large PTSD-focused neuroimaging data set to investigate genomic markers for association to cortical and subcortical volumes such as hippocampus, amygdala, and medial prefrontal cortex structures as well as regional cortical thickness changes that are associated with PTSD. Genomic markers found to predict imaging QTs may have a role in PTSD symptoms or diagnoses (Meyer-Lindenberg and Weinberger, 2006).

DISCUSSION

There are several ways in which the PGC-PTSD will represent and advance the current cutting-edge of PTSD genetics research. First and foremost, the PGC-PTSD will build on what the PGC has learned in other disease domains. We believe that the PGC-PTSD, through its investigation of genetic variation, epigenetic variation, and neuroimaging characteristics of PTSD will provide new and important insights into the biological underpinnings of PTSD risk. The PGC-PTSD additionally has the goal of developing clinically useful biomarkers of PTSD. The work of the PGC-PTSD will inform the development of at least three types of clinical biomarkers. The first are predictive biomarkers that reliably distinguish persons at high *vs* low risk for the development of PTSD following trauma. A gene or combination of genes associated with PTSD may, in conjunction with other information, contribute to an algorithm for estimating the risk of developing PTSD. Such a risk algorithm could be used in first-response settings or the military to better target preventive interventions.

The second type of biomarker likely to be informed by the discoveries of the PGC PTSD working group is related to treatment matching. There are several effective interventions for PTSD including prolonged exposure, cognitive processing therapy, skills training in affective and interpersonal regulation, and pharmacological interventions. However, little is known about which of these treatments might be most effective for which patients. One of the long-term goals of the PGC–PTSD will be to examine whether patients with specific combinations of genetic variants and environmental exposures respond differentially to evidence-based treatments.

The third type of biomarker that may be informed by the work of the PGC–PTSD is relapse prediction. Several of the studies included in the PGC–PTSD meta-analysis are longitudinal and a few are truly prospective (Baker *et al*, 2012; Goldmann *et al*, 2011). Thus, we will be able to examine whether genetic variants associated with PTSD also predict the clinical course of the disorder. If patients with a specific combination of genetic and environmental risk factors are at higher risk of relapse, such patients could be targeted with relapse prevention strategies.

Knowledge of the genetic and environmental architecture of PTSD has the potential to advance our understanding of the pathophysiology of the disorder and inform treatment development. Of particular interest is the development of preventive pharmacological interventions for PTSD that could be administered in the acute aftermath of traumatic events. Many pharmacological agents have been explored in this regard including propranolol and hydrocortisone, but none have shown decisive efficacy for PTSD prevention in large RCTs. The success of GWAS of schizophrenia and bipolar disorder has led to the identification of new treatment targets (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Nurnberger *et al*, 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014). Clinical trials are underway to determine whether these will translate into more effective treatments. Rather than simply generating a list of associated DNA variants, our goal is to produce the same successes for PTSD.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

cal theories involving excessive serotonin have been discussed both in anxiety[3] and depression.[4]

The tracer-specific concerns raised by Dr Jacobsen have been discussed in detail previously.[5,6] Briefly, PET assessment of endogenous serotonin formation assumes that [^{11}C]5-HTP influx rate is a proxy for endogenous amino acid decarboxylase (AADC) activity, being proportional to tryptophan hydroxylase activity. Dr Jacobsen proposes 2 alternatives as to why we found increased AADC activity, both having serotonin "deficiency" in common. Dr Jacobsen argues that increased AADC activity may result from less endogenous 5-HTP competing with the radiotracer. However, as pointed out by Dr Jacobsen, AADC activity is not the rate-limiting step, making it highly unlikely that the very low tracer doses (nanomoles) of [^{11}C]5-HTP injected,[5] corresponding to picomole amounts entering the brain, would cause enzyme saturation. Indeed the Michaelis-Menten constant of AADC (micromoles per liter) mentioned by Dr Jacobsen supports this. In our article,[1] we speculated that downregulation of serotonin-1A autoreceptors disinhibited serotonergic synthesis and firing. Indeed, reduced serotonin-1A receptor binding has been consistently demonstrated in molecular neuroimaging studies of anxiety disorders. Our findings are also in agreement with animal studies reporting anxiogenic effects of serotonin, including higher tryptophan hydroxylase expression and extracellular amygdala serotonin levels in high-anxious Wistar rats compared with low-anxious counterparts.[3] Thus, interpreting increased AADC activity as reflecting serotonin deficiency does not properly account for the existing data.

In the limitations section, we addressed Dr Jacobsen's third alternative (ie, that increased AADC activity may reflect anomalies in nonserotonergic neurons). While it is true that AADC is expressed, for example, in dopaminergic neurons, it should be noted that we found increased [^{11}C]5-HTP influx rate in the raphe nuclei, which contain very few dopaminergic neurons. Also, PET work has shown incomplete overlap between the influx rates of [^{11}C]5-HTP and [^{11}C]DOPA,[7] suggesting at least some degree of separation of the serotonergic from the dopaminergic system using [^{11}C]5-HTP.

We acknowledge the difficulties in interpreting PET data of enzyme activity in terms of excess or deficiency given the brain's compensatory and adaptive functions. Nonetheless, we argue that increased activity in serotonergic neurons is likely reflected in increased AADC activity assessed with [^{11}C]5-HTP, and that our findings are best interpreted as increased serotonin synthesis in SAD.

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Heart Rate Variability and Posttraumatic Stress Disorder

To the Editor We appreciate the thoughtful editorial by Shah and Vaccarino[1] in *JAMA Psychiatry* in response to our prospective investigation of the association of predeployment heart rate variability (HRV) with postdeployment posttraumatic stress disorder (PTSD) in military service personnel.[2] We agree that the findings are in need of replication by other groups, particularly with attention to limitations inherent in HRV indices including the low frequency to high frequency ratio, among others.[3] Shah and Vaccarino[1] mentioned several concerns, briefly addressed here.

The authors highlighted the attrition rate from predeployment to postdeployment (39% in the first phase of the Marine Resiliency Study [MRS-I] and 36% in the second phase of the Marine Resiliency Study [MRS-II]) as a factor limiting causal inference. The *t* test comparisons between participants with and without a postdeployment visit yielded no differences in predeployment HRV, PTSD symptom scores, or Life Events Checklist scores. However, it remains possible that service members who did not return for their postdeployment assessment (eg, left the military following deployment) may have been a group uniquely affected by deployment. The main causes of attrition were deployment-related death, injury of such severity that postdeployment return with the battalion was precluded, or high mobility (eg, change of battalion, assignment to specialized training, discharge from the military, and interference from a civilian work schedule). Of the available participants for follow-up, only a very small number actively declined to participate in the postdeployment assessment (4% in MRS-I and 0.04% in MRS-II). The MRS attrition rates matched other recent longitudinal studies of PTSD in service members (40% in the study by Stein et al[4] and 50% in the study by Polusny et al[5]), with causes of attrition (ie, high mobility) being similar across studies.

The authors also commented on the lack of inclusion of factors such as medical history, health behaviors, depression, and trauma history. Our access to medical health records was incomplete; however, it should be noted that owing to deployment requirements, this was a relatively healthy population. When predeployment Beck Depression Inventory 2 scores were included in the regression model, the normalized low frequency to high frequency ratio retained statistical significance as a predictor of postdeployment PTSD (odds ratio, 1.61; 95% CI, 1.11-2.34; $P = .01$). Similar results were obtained with postdeployment Beck Depression Inventory 2 scores. Likewise, when Life Events Checklist scores were included in the regression, again the low frequency to high frequency ratio retained its significance (odds ratio, 1.61; 95% CI, 1.12-2.30; $P = .01$). Therefore, we argue that predeployment HRV, although very likely to be influenced by a host of vulnerability factors, may nevertheless hold independent value in understanding PTSD risk and resilience. We look forward to replication and extension of these findings, which may ultimately provide new targets for prevention and treatment.

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Editorial Note: This letter was shown to the corresponding author of the original article, who declined to reply on behalf of the authors.

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CORRECTION

Error in Author Affiliations: In the Original Investigation titled "Use of Lithium and Anticonvulsants and the Rate of Chronic Kidney Disease: A Nationwide Population-Based Study,"[1] published online November 4, 2015, and also in the December 2015 print issue of *JAMA Psychiatry*, there was an error in the author affiliations. The fourth affiliation should have read, "Department of Psychiatry, Aalborg University Hospital, Aalborg, Denmark (Licht)." This article was corrected online.

1. Kessing LV, Gerds TA, Feldt-Rasmussen B, Andersen PK, Licht RW. Use of lithium and anticonvulsants and the rate of chronic kidney disease: a nationwide population-based study. *JAMA Psychiatry*. 2015;72(12):1182-1191.

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RESEARCH ARTICLE Open Access

Polymorphisms at the *F12* and *KLKB1* loci have significant trait association with activation of the renin-angiotensin system

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Abstract

Background: Plasma coagulation Factor XIIa (Hageman factor; encoded by *F12*) and kallikrein (KAL or Fletcher factor; encoded by *KLKB1*) are proteases of the kallikerin-kinin system involved in converting the inactive circulating prorenin to renin. Renin is a key enzyme in the formation of angiotensin II, which regulates blood pressure, fluid and electrolyte balance and is a biomarker for cardiovascular, metabolic and renal function. The renin-angiotensin system is implicated in extinction learning in posttraumatic stress disorder.

Methods & Results: Active plasma renin was measured from two independent cohorts- civilian twins and siblings, as well as U.S. Marines, for a total of 1,180 subjects. Genotyping these subjects revealed that the carriers of the minor alleles at the two loci- *F12* and *KLKB1* had a significant association with reduced levels of active plasma renin. Meta-analyses confirmed the association across cohorts. In vitro studies verified digestion of human recombinant pro-renin by kallikrein (KAL) to generate active renin. Subsequently, the active renin was able to digest the synthetic substrate angiotensinogen to angiotensin-I. Examination of mouse juxtaglomerular cell line and mouse kidney sections showed co-localization of KAL with renin. Expression of either *REN* or *KLKB1* was regulated in cell line and rodent models of hypertension in response to oxidative stress, interleukin or arterial blood pressure changes.

Conclusions: The functional variants of *KLKB1* (rs3733402) and *F12* (rs1801020) disrupted the cascade of enzymatic events, resulting in diminished formation of active renin. Using genetic, cellular and molecular approaches we found that conversion of zymogen prorenin to renin was influenced by these polymorphisms. The study suggests that the variant version of protease factor XIIa due to the amino acid substitution had reduced ability to activate prekallikrein to KAL. As a result KAL has reduced efficacy in converting prorenin to renin and this step of the pathway leading to activation of renin affords a potential therapeutic target.

Keywords: FXIIa (active protease encoded by gene *F12* or Hageman factor), Kallikrein/KAL (active protease encoded for by gene *KLKB1* or Fletcher factor), rs3733402, rs1801020, PTSD (posttraumatic stress disorder), Hypertension

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Background

Hypertension is a global public health issue and contributes to the burden of heart disease, stroke, kidney failure and premature mortality (13 % of total deaths worldwide)[1]. The kidney serves as a major organ for maintaining normal blood pressure (BP) and the local renal renin angiotensin system (RAS) pathway acts as the master regulator of renal function during hypertension [2–4]. The renin-angiotensin-aldosterone system (RAAS) is a signaling pathway responsible for regulating the body's blood pressure [5–8]. Stimulated by low BP the kidney releases renin, this triggers a signal transduction pathway generating eventually angiotensin II that causes vasoconstriction, leading to increase in BP. Several cardiovascular therapies for high BP, target the RAAS system and these therapies are now being explored for their efficacy in treating PTSD [9, 10].

The juxtaglomerular (JG) cells in the kidney express renin a member of the aspartyl protease family. It is the limiting enzyme in RAS pathway that converts angiotensinogen to angiotensin I (Ang I) [11]. Renin production is tightly regulated at the transcriptional level and the active renin is released into the circulation through regulated exocytosis [11, 12]. About 80 % of the renin present in plasma is in an enzymatically inactive form called pro-renin. Kidney processes inactive pro-renin to renin and is the major source of circulating active renin in humans. The plasma renin concentration contributes significantly to cardiovascular and renal diseases like hypertension, coronary heart disease, and chronic kidney disease [13]. Thus the conversion of pro-renin to renin is a potential regulatory site for therapeutic intervention.

We studied the effect of the *KLKB1* (located on chromosome 4) missense variant rs3733402 (Asn124Ser) on circulating levels of active renin and observed that homozygous carriers of the minor allele (Ser/Ser) displayed lower levels of active renin [14]. In vitro proteolysis and cell biology indicated that pro-renin was a substrate for plasma kallikrein (KAL). The KAL-activated renin in turn, was able to cleave substrate angiotensinogen to angiotensin 1 the precursor for vasoconstrictor angiotensin II. Situated on chromosome 5, the coagulation factor *F12* 5'-UTR variant rs1801020 also showed significant association with plasma levels of active renin. The *F12* locus encodes for the FXIIa protease responsible for converting pre-kallikrein to KAL. The possible implication of the intrinsic coagulation system and the fibrinolytic system in renin activation has been discussed. In both the independent cohorts a strong association was observed between levels of active renin and occurrence of the minor alleles.

Methods

Twin and sibling subjects

Twin and sibling participants (TSP) for the human study were recruited from southern California by access to a population birth record-based twin registry [15], as well as by newspaper advertisement [16]. The University of California San Diego, Institutional Review Board provided approval for the study and each subject or the parent of the minor subjects, gave written informed consent. A subset of 381 individuals of the TSP population was randomly selected and included 60 dizygotic (DZ) and 160 monozygotic (MZ) twin pairs. Zygosity of twins was confirmed genetically by use of microsatellite and single nucleotide polymorphism (SNP) markers [16]. Initially ethnicity was established by self-identification, including information on geographic origin of both parents and all four grandparents, and only individuals of Caucasian or Hispanic ancestry/ethnicity are included here. The age of the subjects ranged from 14 to 78 years, with a median of 39. Phenotyping (biochemical and physiological) was conducted as previously described [16]. All of the 381 TSP subjects with both genotypes and phenotypes were included in the analyses (see below).

Molecular genetics, genomic DNA and genotyping

Genomic DNA was extracted from leukocytes in EDTA-anticoagulated blood after proteinase-K digestion of proteins, by adsorption/elution from Qiagen columns, as previously described [16], and genotyped for 592,312 SNPs using the Illumina 610-Quad genotyping array and passed final quality control (QC: see below). For each MZ twin pairs, only one individual underwent GWAS, and the genotype information was used for both members of MZ twins. During analysis, family structure was accounted for in MERLIN (see below).

Biochemical assay of active renin in human plasma

EDTA–anticoagulated plasma samples were collected from seated subjects, and stored frozen at −70 °C until assayed. Circulating active renin was quantified at room temperature for 3 hours with a 2-site IRMA [17] wherein the mouse monoclonal anti human renin antibody was specific for a renin epitope formed after excision of active renin from pro-renin (DSL, Webster, TX; DSL-25100); the active renin assay sensitivity was ~0.48 pg/ml, with intra-assay coefficients of variation from 1.4-4.3 %, and inter-assay coefficients of 1.9-3.0 %.

Genetic association analyses

To test SNP on phenotype effects with explicit accounting for family structure for the TSP cohort, MERLIN v1.1.2 (http://www.sph.umich.edu/csg/abecasis/merlin/) was used. As an additional QC step, unlikely genotypes based on expected inheritance patterns were removed using Merlin's Pedwipe procedure. A maximum likelihood estimation test of a variance components model was used, incorporating a variance-covariance matrix that allows for family relatedness, including twin status,

to be modeled and appropriately controlled for in the association test. In addition, age, gender, and the first MDS component were included as covariates. A standard criterion of $p < 5x10^{-8}$ across the genome was used to indicate significance of single SNPs on traits. The "Manhattan" plots visualized results across the genome, as well as local "SNAP" (SNP Annotation and Proxy Search) plots [18] <http://www.broadinstitute.org/mpg/snap/ldplot.php>.

Replication Marine Resiliency Study (MRS)

We also measured active plasma renin (by ELISA) in samples from 799 healthy unrelated male Marines from the Marine Resiliency Study (MRS) with available genotypes [19]. The method for genotyping of MRS subjects has been detailed earlier [20, 21]. In brief, genotyping was carried out using the HumanOmniExpressExome (HOEE) array with 951,117 loci from Illumina (http://www.illumina.com/), resulting in a high initial locus success rate and overall data quality. Additional data cleaning was performed in PLINK v1.07 [22], using standard procedures. All subjects included here were active duty male and of European ancestry [23]. All subjects provided written consent for the genetic study. Association of plasma renin activity with genotypes were performed using a linear regression in PLINK (v.1.07) using age and 3 principal components (PC's) to correct for population stratification as covariates. We used the Genetic Power Calculator from Purcell et al. to estimate power [24]. Based on an effect size estimate of 1 % of variance explained by a candidate variant, we estimate that we had 83 % power to detect an effect of SNP on renin levels at an alpha level of 0.05, given the number of samples available in the MRS. Furthermore we estimate that we would have >94 % power to detect an effect of this size in a meta-analysis of the MRS and TSP.

Meta-analyses

Results from the TSP and MRS data were combined in an inverse variance and weighted fixed-effect meta-analysis was carried out using METAL [25].

Protein chemistry and enzymology

Digestion of recombinant human pro-renin by human KAL

Recombinant human pro-renin (5 μM) (Cayman Chemical, catalog number 10007599) was digested with protease human KAL (kallikrein, human plasma, Calbiochem, EMD Millipore, catalog number 420307, specific activity 15 U/mg protein) (1 μM) at 37 °C for 15 min in 12 μl of reaction volume with assay buffer (50 mM Tris, pH 7.5, NaCl 250 mM). The reaction was terminated by adding aprotinin (2 μM), purified by ZipTip (small C-18 column) and then analyzed by MALDI-TOF. For SDS-PAGE, pro-renin was incubated in absence or presence of KAL as mentioned above for 2 hours, and analyzed on 10 % or 4-12 % (gradient) NuPAGE gels.

Digestion of renin substrate angiotensinogen (AGT) with KAL-activated renin

Human pro-renin (5 μM) was digested with KAL (1 μM) in 50 mM Tris, pH 7.5 and NaCl 250 mM in a volume of 12 μl for 15 min at 37 °C, as mentioned above in the first step. In the second step, 12 μl of sodium acetate buffer, pH 5.5 containing angiotensinogen synthetic tetradecapeptide (14 amino acids; DRVYIHPFHL↓VIHN) (Phoenix Pharmaceuticals, Inc.) was added (in final concentrations of sodium acetate 0.2 M and tetradecapeptide 10 μM), and further incubated for another 15 min at 37 °C. The reaction digests were then purified through ZipTip adsorption/elution, and were analyzed by MALDI-TOF.

MALDI-TOF analysis

MALDI-TOF analyses were performed as described before using a PE Biosystems Voyager DeSTR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) [26]. Resulting peptide masses were analyzed in the Protein-Prospector Program (<http://prospector.ucsf.edu>) to identify the possible fragments of the respective proteins.

Identification of active renin and pro-renin protein bands in KAL digests, analysis by LC-MS/MS sequencing

Gel slices were cut, processed for in-gel trypsin digestion and the extracted peptides were analyzed by reverse-phase liquid chromatography (LC) in combination with tandem mass spectrometry using electrospray ionization with a QSTAR-Elite hybrid mass spectrometer (AB/MDS Sciex) as described before [27]. Peptide identifications were made using the Paragon algorithm executed in Protein Pilot 2.0 (Life Technologies).

Amino acid sequence analysis by TOF/TOF

Tandem mass analysis (MS/MS) for sequencing was performed on a 4800 MALDI-TOF-TOF mass spectrometer (Applied Biosystems) as described before [26].

Mouse juxtaglomerular cell culture

Mouse kidney juxtaglomerular cells As4.1 (ATCC * CRL-2193™) were grown in DMEM high-glucose (GIBCO) with 10 % FBS and Penicillin/streptomycin/glutamine media at 37 °C with 5 % CO_2.

Co-localization of Renin and KAL by immunofluorescence

Mouse CRL-2193 (As4.1) juxtaglomerular cells

Cells were grown on cover slips, washed with PBS and were fixed with 2.5 % paraformaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized with 0.5 % Triton in PBS for 10 min at room

temperature. Cells were blocked using 5 % BSA in PBS for 30 min followed by primary antibody incubation [rabbit anti KAL (1:100, Bioss) and goat anti renin (1:100, Santa Cruz Biotechnology)] in 2 % BSA for 2 hr at room temperature. Coverslips were washed 3 times 5 min each and then incubated with secondary antibody Alexa Fluor 488 nm (green) coupled to donkey anti rabbit (1:250, Invitrogen) and Alexa Fluor 594 nm (red) donkey anti goat (1:350, Invitrogen) along with Hoechst 33342 (nuclear stain; 1 μg/mL) in 1 % BSA for 1 hr at room temperature. Coverslips were washed and mounted on glass slide using Slowfade-antifade (Molecular Probes). Images were acquired on a Delta Vision deconvolution microscope and SoftWorx software (Applied Precision, Issaquah, WA), using 60x objective as described previously [28].

Mouse kidney immunohistochemistry

Formaldehyde-fixed paraffin-embedded kidney tissue sections were cleared of paraffin and hydrated through graded alcohol and boiled in 100 °C for 20–30 min for antigen retrieval [29]. After permeabilization and blocking, sections were incubated overnight at 4 °C with primary antibodies to renin and KAL, followed by incubation with Alexa Fluor secondary antibodies as described above. Images were captured on a Delta Vision deconvolution microscope using 20x objective.

REN and KLKB1 mRNA expression in organs and cells

Transcriptomes of mouse adrenal gland from mouse strains blood pressure high (BPH) and blood pressure low (BPL) (each in triplicate) [30]; rat adrenal gland (SHR and WKY strains, each in triplicate) [31] and mouse As4.1 juxtaglomerular cells (in duplicate) [32] were profiled by microarray analysis as previously described, and data are available at NCBI GEO. Data were globally normalized to median expression, and then analyzed statistically.

Statistical analyses

The results were expressed as mean ± one SEM. Multiple comparisons were made using one-way ANOVA followed by Bonferroni post hoc tests, or by two-way ANOVA using Kaleidagraph (Synergy Software, Reading, PA). Statistical significance was concluded at $p < 0.05$.

Results

Meta-analysis of genetic association for polymorphisms at the *F12* and *KLKB1* loci and active renin concentration in plasma

The best-characterized functional polymorphism at the *KLKB1* locus rs3733402 results in loss-of-function amino acid substitution Asn124Ser [33]. This substitution in the apple 2 domain impairs binding and digestion of the classical substrate HMWK (high molecular weight kininogen) [14]. At the *F12* locus, the rs1801020 polymorphism is in the 5'-UTR (C46T) creates a new upstream translational start codon, thereby attenuating formation of the authentic F12 protease [34].

Since these proteases are part of the kallikrein-kinin system and interact with each other at the molecular level, we looked at genetic association of the polymorphisms described above with levels of active renin in plasma. The effect of the human polymorphisms rs3733402 in the *KLKB1* locus and the rs1801020 in *F12* locus were very significant on the active renin levels in plasma of both the TSP and MRS populations (Table 1, Fig. 1). In both cases, minor alleles were associated with low levels of active renin in the plasma (Fig. 1). Meta-analysis combining the TSP and one independent population (MRS) for a total of $n = 1{,}180$ subjects, indicated allelic effects consistent in magnitude (beta, or effect size per allele) and direction (sign on slope) across populations. The overall slope of the meta-analysis regression for rs3733402 and rs1801020 was beta = 0.055 and 0.057, with SE =0.014 ($p = 6.83 \times 10^{-5}$) and = 0.016 ($p = 0.0003$) respectively (Table 1).

Table 1 Meta-analysis of the effect of *KLKB1* and *F12* genetic polymorphisms on generation of active renin in human plasma

KLKB1 (rs3733402)														
Cohort	A1	A2	N	BETA	SE	P	MAF	HetISq	HetP	G/G Freq	G/A Freq	A/A Freq	HWE chi-square	HWE p
TSP	G	A	381	−0.071	0.025	0.005	0.5			20.42%	49.17%	30.42%	0.399	0.712
MRS	G	A	799	−0.048	0.016	0.0037	0.47			20.40%	52.82%	26.78%	2.67	0.102
Meta-analysis	G	A	1180	−0.055	0.014	7.22E-05	0.48	0	0.507					
F12 (rs1801020)														
Cohort	A1	A2	N	BETA	SE	P	MAF	HetISq	HetP	A/A Freq	A/G Freq	G/G Freq	HWE chi-square	HWE p
TSP	A	G	381	−0.061	0.031	0.0459	0.23			4.58%	33.75%	61.67%	0.514	0.426
MRS	A	G	798	−0.055	0.018	0.0026	0.24			6.14%	35.71%	58.15%	0.339	0.0561
Meta-analysis	A	G	1179	−0.057	0.016	0.0003	0.24	0	0.947					

A1/A2: effect allele/non-effect allele, N: sample size, BETA: estimated beta coefficient, SE: standard error of beta, P: p-value for beta, MAF: minor allele frequency, HetP: p-value for Cochran's Q statistic, HetISq: I^2 heterogeneity index, TSP: twin & sibling participants, MRS: Marine resiliency study

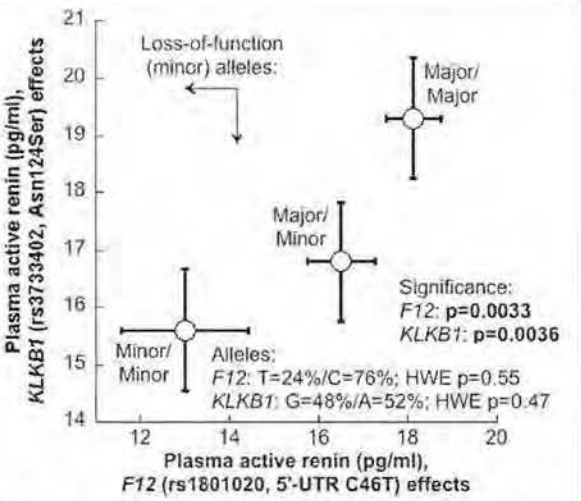
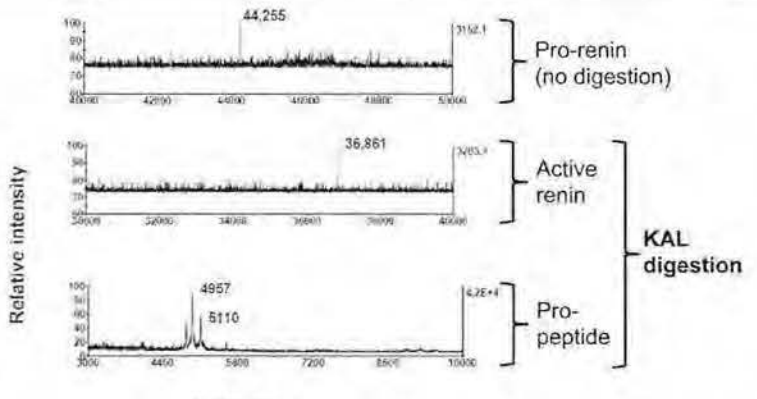


Fig. 1 Effect of polymorphisms in the *KLKB1* (Asn124Ser) and *F12* (5' UTR C46T) loci on circulating levels of active renin in UCSD twins/siblings and in MRS subjects. Polymorphisms at *F12* and *KLKB1* loci influenced plasma active renin levels in two independent cohorts civilian and marines. Each SNP was in Hardy-Weinberg equilibrium with $p > 0.05$

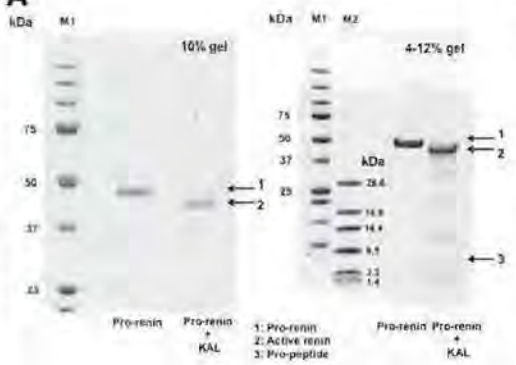
Digestion of human recombinant pro-renin with kallikrein (KAL) yields active renin and the pro-peptide byproduct

MALDI-TOF analysis of KAL digested pro-renin displayed two peaks of m/z 36,861 and 5100, corresponding to the theoretical masses of active renin and pro-peptide respectively (Fig. 2, lower panels). In control reaction, where pro-renin was incubated in absence of KAL, MS chromatogram showed a single peak of m/z 44,255, representing the intact pro-renin (Fig. 2, upper panel). In order to identify the sequence of the digested products, the digestion mixture was subjected to SDS-PAGE on a 10 % gel to separate high molecular weight pro-renin and active renin, and on a 4-12 % gradient gel to separate low molecular weight pro-peptide fragment. A faster migrating band compared to that of pro-renin appeared only in the KAL digested sample (Fig. 3a, marked with arrow 2). Generation of a low molecular weight fragment of ~ 5 kDa was evidenced after digestion of pro-renin with KAL (Fig. 3a, right panel, marked with arrow 3). Fragments marked with arrow 2 and 3 were cut out from the gel, trypsin digested and subjected to LC-MS analysis for identification. Peptides identified from gel fragment 3 showed significant coverage on the N and C-terminal of pro-peptide sequence (Fig. 3b), whereas same from gel fragment 2 showed coverage on active-renin (Fig. 3b). Since LC-MS analysis from gel fragment 3 identified some active renin sequence and gel fragment 2 identified some pro-peptide sequence, we quantified the data by normalizing the total sum of spectra for pro-peptide and active renin observed in gel fragment 2 and 3 by their amino acid length. Quantification of mass spec data showed a significant enrichment (400-fold) of pro-peptide to active renin ratio in gel fragment 3 over gel fragment 2 (Fig. 3c).



Target	m/z Observed	m/z Expected	Deviation (%)
Pro-renin	44,255	42,324	4.6
Active renin	36,861	37,237	1.0
Pro-peptide	5110	5105	0.1

Fig. 2 Mass spectrometric analysis of the KAL digested samples of recombinant pro-renin. Recombinant pro-renin was incubated in absence (upper panel) or presence (middle and bottom panel) of KAL in the assay buffer as mentioned before. The digestion mixture was acidified and purified through ZipTip and subjected to MALDI-TOF analysis in linear mode. Observed masses were compared with the theoretical mass predicted by ProteinProspector program and are shown in the table



B

Cleavage of human Pro-renin by KAL:
Peptide coverage during LC-MS/MS.
Peptides identified by MS/MS in **bold** type.
Template: UniProtKB P00797 (RENI_HUMAN).
Pro-Peptide:
LPTDTTTFKRIFLKRMPSIRESLKERGVDMAR**LGPEWSQPMKR**
Active-Renin:
LTLGNTTSSVILTNYMDTQYYGEIGIGTPPQTFKVVFDTGSSNVWVPSSKCSRLYTACVY
HKLFDASDSSSYKHNGTELTLRYSTGTVSGFLSQDIITVGGITVTQMFGEVTEMPALPFM
LAEFDGVVGMGFIEQAIGRVTPIFDNIISQGVLKEDVFSFYYNRDSENSQSLGGQIVLGG
SDPQHYEGNFHYINLIKTGVWQIQMKGVSVGSSTLLCEDGCLALVDTGASYISGSTSSIE
KLMEALGAKKRLFDYVVKCNEGPTLPDISFHLGGKEYTLTSADYVFQESYSSKKLCTLAI
HAMDIPPPTGPTWALGATFIRKFYTEFDRRNNR**IGFALAR**

C **Quantification of Mass spec data. Gel bands 2 and 3 were subjected to trypsin digestion and LC-MS/MS analysis. Total Sum represents the sum of each peptide hit with confidence 99 %.**

	Column-1	Column-2	Column-3	Column-4	Column-5	Column-6	Column-7
Row-1	Target	Amino acid length	Total Sum of pro-peptide spectra observed	Total Sum of Active renin spectra observed	Pro-peptide (P) (Amino acid length normalized spectral count) (=C3R2/C2R2)	Active renin (A) (Amino acid length normalized spectral count)	P/A ratio
Row-2	Pro-peptide	43	130	26	3.02 (=C3R2/C2R2)	.076 (=C4R2/C2R3)	39.5
Row-3	Active renin	340	8	646	.186 (=C3R3/C2R2)	1.9 (=C4R3/C2R3)	.097

Fig. 3 Molecular mass analysis of fragments generated by KAL digestion of pro-renin; identification of active renin and pro-peptide fragments. **a** Recombinant pro-renin was digested with KAL as described in Fig. 2 and subjected to SDS-PAGE; 10 % gel (left panel) for separation of active renin from pro-renin and 4–12 % gel (right panel) for identification of lower (~5 kDa) fragment). Fragments were numbered as 1 (pro-renin), 2 (active renin) and 3 (pro-peptide). M1 and M2 are SDS-PAGE molecular weight standards in broad range and low range respectively **b** Fragment 2 and 3 were cut out and subjected to trypsin digestion and LC-MS/MS analysis separately for identification. Peptide coverage (bold type) identified by MS/MS in the termini of pro-peptide (from fragment 3) and active renin (from fragment 2) is shown. **c** Quantification of the MS data. The total sum of pro-peptide and active renin spectra observed were normalized by the amino acid length (43 for pro-peptide and 340 for the active renin) to represent the pro-peptide to active renin ratio in gel fragments 2 or 3

KAL digested pro-renin cleaves angiotensinogen substrate to generate angiotensin I

Active renin digests substrate angiotensinogen to generate angiotensin I (Ang I). We tested the ability of KAL digested pro-renin to digest angiotensinogen. The pre-angiotensinogen 1–14 tetra deca peptide (AGT) was incubated with the KAL-digested pro-renin. Analysis of the digestion reaction containing AGT and KAL revealed one major peptide of m/z 1759.9 (Fig. 4a, upper panel). Incubation of pro-renin with KAL followed by the addition of AGT generated a major peak of m/z 1296.81 (Fig. 4a, lower panel). MS/MS analysis of the precursor mass 1759.9 and 1296.8 confirmed the sequence of these two peptides as amino acids 34–47 and 34–43 of human angiotensinogen (Fig. 4b). Quantification of MS data suggest ~ 96 % generation of Ang I peptide in reaction containing KAL, pro-renin and AGT, whereas only 24 % in presence of pro-renin and AGT and 8 % in presence of KAL and AGT. The generation of Ang I or AGT 1–10 peptide of m/z 1296.8 was not detected in digestion reactions containing only KAL, pro-renin, AGT or in KAL and pro-renin combination (Fig. 4c).

Renin co-localized with KAL, in kidney JG cells and their renin secretory granules

Immunofluorescence experiments of mouse juxtaglomerular cells (Fig. 5a) as well as in mouse kidney section (Fig. 5b), was used to establish renin's subcellular co-localization with its processing enzyme KAL. The immunofluorescence micrographs showed that renin and KAL co-localized partially as evidenced by the orange/yellow fluorescence in the overlay figures. Pearson coefficient of co-localization was 0.15 for As1.4 cells and 0.5 for the kidney section.

Endogenous expression of *KLKB1* and *REN*

After confirming by in vitro assay that KAL processed prorenin to active renin, we analyzed how the expressions of *KLKB1* and *REN* genes might be correlated

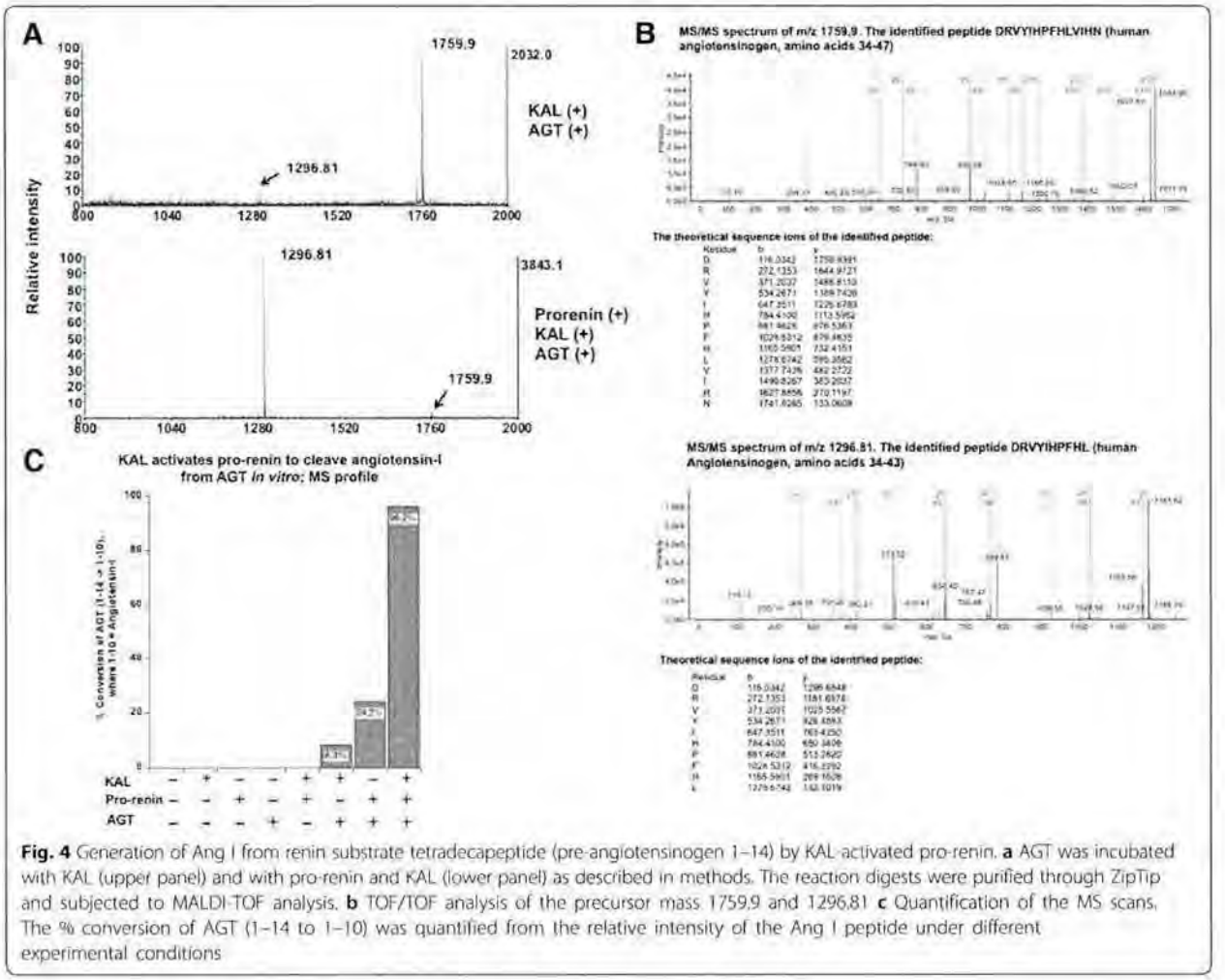


Fig. 4 Generation of Ang I from renin substrate tetradecapeptide (pre-angiotensinogen 1–14) by KAL-activated pro-renin. **a** AGT was incubated with KAL (upper panel) and with pro-renin and KAL (lower panel) as described in methods. The reaction digests were purified through ZipTip and subjected to MALDI-TOF analysis. **b** TOF/TOF analysis of the precursor mass 1759.9 and 1296.81 **c** Quantification of the MS scans. The % conversion of AGT (1–14 to 1–10) was quantified from the relative intensity of the Ang I peptide under different experimental conditions

under various physiological conditions. *REN* and *KLKB1* mRNA expression data were collected and analyzed for in mouse As4.1 cells (Fig. 6a) and adrenal tissues of rodent genetic hypertension models: blood pressure low (BPL) and blood pressure high (BPH) mouse models and normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rat models (Fig. 6b). In As4.1 cells feedback inhibition of renin expression was observed by the addition of interleukin 1-β or hydrogen peroxide, concomitantly KLKB1 expression remained unaltered. The hypotensive phenotype of BPL mice triggered renin expression, ~ 4 fold higher compared to hypertensive BPH mice. However the expression of KLKB1 did not differ significantly amongst BPL and BPH mice. The normotensive WKY rats have significantly higher *KLKB1* expression (~5.5 fold) compared to the hypertensive SHR rats with more or less similar level of REN mRNA expression in both rat models. Thus regulation of blood pressure under various physiological conditions may involve modulation in the expression of either *REN* or *KLKB1*.

Discussion

In vitro studies have demonstrated that proteases such as trypsin, plasmin, pepsin, kallikrein and several others activate zymogen pro-renin to active renin [35–38]. Studies before the era of mass spectroscopy suggested involvement of KLKB1 and FXIIa in pro-renin processing [39–41]. Genetic variation at the *KLKB1* locus (encoding for plasma pre-kallikrein or Fletcher factor; EC 3.4.21.34) was previously most widely investigated for its roles in coagulation and allergy. We demonstrate using in vitro enzymatic assay the ability of active protease KAL in processing pro-renin → renin. A second association of renin activity and the protease *F12* locus (encoding for Factor XII or Hageman factor; EC 3.4.21.38) suggests a cascade of enzymatic events

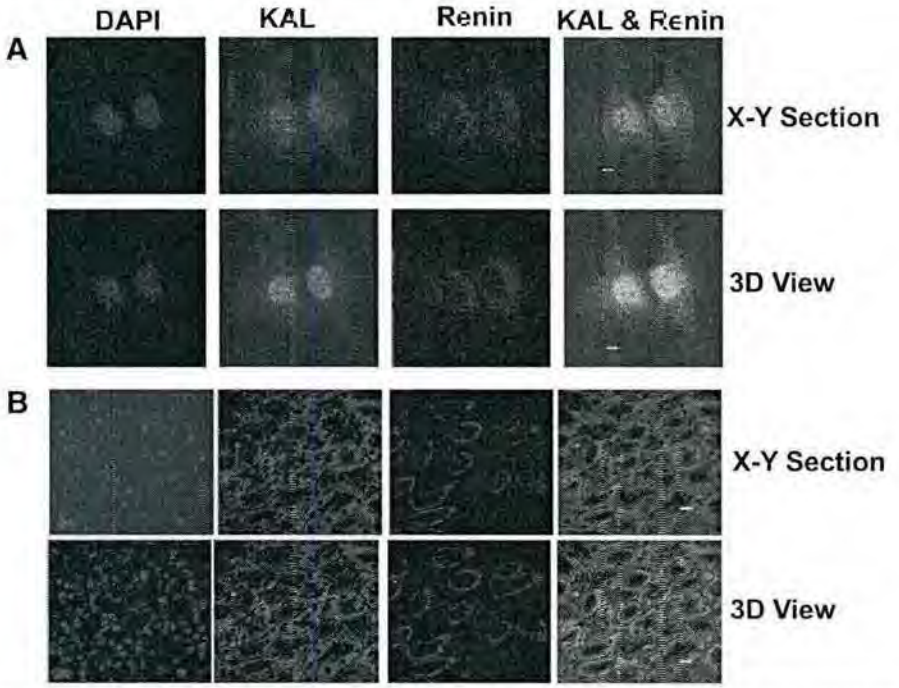


Fig. 5 Co-localization of renin and KAL in **a** As4.1 (CRL-2193) juxtaglomerular cells and in **b** mouse kidney. The cells (upper two panels) and kidney sections (lower two panels) were stained with rabbit anti-KAL primary and donkey anti-rabbit IgG- Alexa Fluor®488 conjugated secondary (green); and goat anti-renin primary and donkey anti-goat IgG- Fluor®594 conjugated secondary (red). The nuclei were visualized with Hoechst 33342 dye (blue). A series of *xy* optical sections along the *z*-axis was acquired using a 60x oil immersion objective for cells and 20x objective for kidney section by deconvolution microscopy and the data set was processed to generate three-dimensional (3D) or representative *xy* section. Co-localization of KAL (green) and renin (red) is shown by overlay of the images (yellow fluorescence). Bar scale is 5 μM

(FXIIa → KAL) in control of pro-renin activation. Generation of active renin by the cascade thus provides evidence of a site for BP regulation.

The *KLKB1* locus lies directly beneath a previously described LOD peak (LOD = 3.2) for BP on chromosome 8 in the genetically hypertensive strain of mice (BPH) [42]. We therefore explored the effects of *KLKB1* genetic variation upon formation of active renin. While most of the *KLKB1* single nucleotide polymorphisms (SNPs) reported are located in the non-coding regions, rs3733402

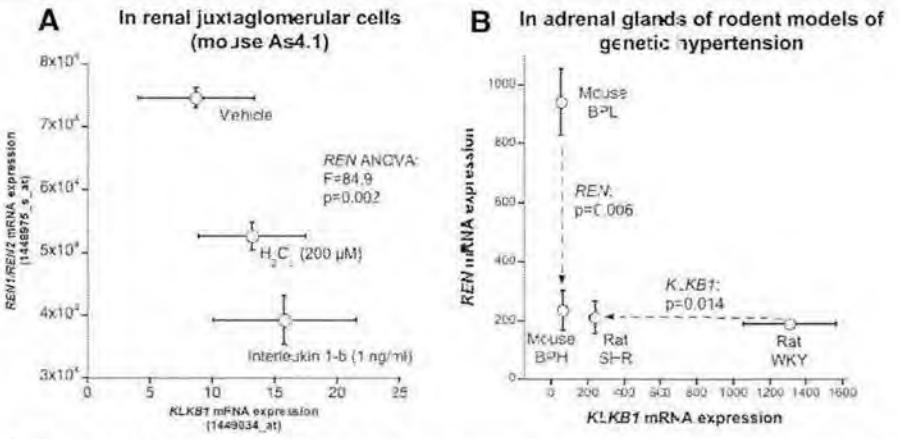


Fig. 6 Correlation of *REN* and *KLKB1* mRNA expression in **a** renal juxtaglomerular cells and (B) in the adrenal glands of rodent models of genetic hypertension (mouse: BPH/BPL and rat: SHR/WKY). *REN* and *KLKB1* mRNA expression data were collected and compared for (**a** and **b**)

in exon 5 results in an amino acid substitution Asn124Ser [14, 33]. This mutation in the apple domain 2 of heavy chain reduces the binding of KAL to its substrate HMWK, and therefore this SNP was chosen to investigate its association with prorenin processing. Indeed, an immunoassay specific for active renin revealed that Ser/Ser homozygotes had lower circulating active renin (Fig. 1), consistent with diminished pro-renin cleavage by a less active Ser allele. Previously, rs3733402 has shown strong association with pre-pro-endothelin-1 and pre pro-adrenomedullin in the Prevention of Renal and Vascular End stage disease (PREVEND) study [43]. In the recent study by Lieb et al. the top SNPs identified were rs12374220, an intronic variant in the *TENM3* gene, rs5030062 in the intron 6 of kininogen 1 gene and rs4253311 in intron 11 of the kallikrein B (*KLKB1*) gene. The intronic SNP rs4253311 provided no evidence for association with renin concentrations and explained 0.87 % of plasma renin activity variance [44]. In our study MALDI mass spectrometry documented the formation of active renin and the pro-peptide after digestion of pro-renin with KAL (Fig. 2 & Fig. 3). Furthermore the sub-cellular co-localization of renin with KAL suggests molecular interaction between these two proteins (Fig. 5a &b). Renin immunoreactivity has previously been shown in the cytoplasmic granules of cultured JG cells and in kidney sections [45]. The cleavage sites involved in pro-renin processing include lysine-arginine, which is the recognition site of plasma kallikrein [46]. Our genetic and biochemical data suggests an enzyme-substrate relation between KAL and prorenin. This suggests the possible existence of feedback regulation at the molecular level in the events leading to active renin generation by KAL and BP regulation.

KAL is a glycoprotein that takes part in the surface dependent activation of blood coagulation, fibrinolysis and kinin generation. It is synthesized in the liver and secreted into the blood as prekallikrein, which is then converted to active plasma kallikrein by factor FXIIa [47]. The C46T 5'-UTR polymorphism associated with Hageman factor has been described to be associated with its plasma concentration and thrombotic risk [48, 49]. The KAL protease might catalyzes the conversion of HMWK to bradykinin in one hand, and the active renin on other hand. The downstream target angiotensin converting enzyme (ACE) then modulates the concentration of angiotensin II, the key player of the RAAS system, and bradykinin, a component of the kallikrein-kinin system in opposite direction, therefore establishing a direct interaction between kallikrein-kinin and renin-angiotensin system [50, 51].

The genetic variation in the *F12* and *KLKB1* loci directly affecting their amino acid sequence could ultimately influenced the processing, secretion or circulation of the active renin protein, which in turn mediates the BP phenotype. Allelic effects might also act on the cluster of characteristics associated with cardiovascular risk for which plasma renin is a biomarker. In the coagulation system, it has been reported that even the homozygous deficiency of the *KLKB1* loci results in no discernible coagulopathy [52]. In treatment of hereditary angioedema inhibition of KAL does play a beneficial role, perhaps by inhibition of bradykinin formation [53].

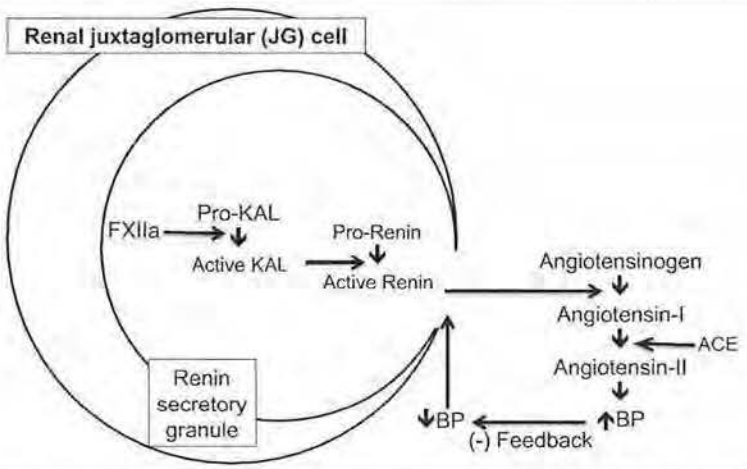


Fig. 7 Hypothetical schematic representing activation of pro-renin by a proteolytic enzyme cascade of FXIIa → KAL, with consequences for regulation of BP. Pro-renin processing within the secretory granule of renal juxtaglomerular cells by sequential enzymatic events catalyzed by F12 and KAL

Advantages and limitations

Here we report a comprehensive GWAS showing correlation between polymorphisms at two independent loci (*KLKB1* rs3733402 and *F12* rs1801020) and plasma renin activity. Cellular and biochemical evidence is provided to establish that correlation. To our knowledge this is the first report of SNPs in two independent loci with significant trait association with activation of renin-angiotensin system. This study focused on the best characterized SNP (rs3733402) in the exon 5 of *KLKB1* gene. Although association of kallikrein with renin activation has previously been described, adequate information on direct in vitro protease biochemistry was lacking. Therefore we used a mass spectrometry approach to characterize in vitro digestion of prorenin by KAL to reestablish kallikrein association with prorenin processing. In the scenario of this genetic association, the efficacy of digestion of prorenin by mutant KAL (Asp124Ser) needs to be compared with that of the wild type KAL. We have not addressed in these populations the active plasma renin association with the previously described intronic variant at *KLKB1* (rs4253311) and other SNPs. Future studies will explore the association of these two SNPs with BP, renal and/or metabolic traits.

Conclusion

Our findings draw attention to the role of KAL as a pro-renin convertase and suggest a potential target for inhibition of the rate-limiting step in the RAS pathway. Polymorphisms at the *KLKB1* (rs3733402) and *F12* (rs1801020) loci are associated with low active plasma renin activity. Genetic, cell and biochemical studies suggest a cascade of enzymatic events involving factor FXIIa activation of prekallikrein to active kallikrein in control of pro-renin activation. Thus plasma kallikrein presents potential as novel therapeutic target for blood pressure regulation with implications of KAL inhibition for treatment of hypertension (Fig. 7).

Abbreviations

AGT: Angiotensinogen; Ang I: Angiotensin I; Ang II: Angiotensin II; BP: Blood pressure; Factor XII (FXII Hageman factor): protein encoded by gene *F12*; JG: Juxtaglomerular; KAL: Kallikrein; MAF: Minor allele frequency; MALDI-TOF: Matrix Assisted Laser Desorption Ionization/Time Of Flight; Prekallikrein (prokallikrein Fletcher factor): protein encoded by gene *KLKB1*; RAAS: Renin-angiotensin-aldosterone system; SNPs: Single nucleotide polymorphisms; TSP: Twin and sibling participants.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NB carried out the biochemical and cell biology studies & wrote the manuscript; AXM, FR,KZ did the genetic and statistical analysis; SAM did immunofluorescence and manuscript preparation; SK,MM,KZ &CMH did the biochemical assays; RSF did the transcriptome studies; SKM helped in the experimental studies. DGB and CMN did the human studies and helped write the manuscript. SV helped in study design, experimentation and writing of the manuscript; DTOC conceived of the study, its design and coordination and helped to draft the manuscript.

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DEPRESSION AND ANXIETY 33:192–202 (2016)

Research Article

HIGH AND LOW THRESHOLD FOR STARTLE REACTIVITY ASSOCIATED WITH PTSD SYMPTOMS BUT NOT PTSD RISK: EVIDENCE FROM A PROSPECTIVE STUDY OF ACTIVE DUTY MARINES

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Background: *Heightened startle response is a symptom of PTSD, but evidence for exaggerated startle in PTSD is inconsistent. This prospective study aimed to clarify whether altered startle reactivity represents a trait risk-factor for developing PTSD or a marker of current PTSD symptoms.* **Methods:** *Marines and Navy Corpsmen were assessed before (n = 2,571) and after (n = 1,632) deployments to Iraq or Afghanistan with the Clinician-Administered PTSD Scale (CAPS). A predeployment startle-threshold task was completed with startle probes presented over 80–114 dB[A] levels. Latent class mixture modeling identified three growth classes of startle performance: "high," "low," and "moderate" threshold classes. Zero-inflated negative binomial regression was used to assess relationships between predeployment startle threshold and pre- and postdeployment psychiatric symptoms.* **Results:** *At predeployment, the low-threshold class had higher PTSD symptom scores. Relative to the moderate-threshold class, low-threshold class membership was associated with decreased likelihood of being symptom-free at predeployment, based on CAPS, with particular associations with numbing and hyperarousal subscales, whereas high-threshold class membership was associated with more severe predeployment PTSD symptoms, in particular avoidance. Associations between low-threshold membership and CAPS symptoms were independent from measures of trauma burden, whereas associations between high-threshold membership and CAPS were not. Predeployment startle threshold did not predict postdeployment symptoms.* **Conclusions:** *This study found that both low startle threshold (heightened reactivity) and high startle threshold (blunted reactivity) were associated with greater current PTSD symptomatology, suggesting that startle reactivity is associated with current PTSD rather than a risk marker for developing PTSD. Depression and Anxiety*

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Key words: *PTSD; startle; combat; risk factor; anxiety; longitudinal study; prospective study*

PTSD is common in veterans across war eras; current prevalence estimates include 12.2% (Vietnam War),[1] 10.1% (Persian Gulf War),[2] and 23% (Operation Enduring Freedom/Operation Iraqi Freedom)[3] and are higher for combat veterans, who have a 1.5–3.5-fold increased risk for PTSD relative to nondeployed veterans.[4] Although evidence-based treatments for PTSD exist, only 40–50% of patients are treatment responsive.[5–8] Mixed treatment response rates may be partly due to heterogeneity of symptoms and underlying pathology.[9] Both treatment and prevention strategies might be improved by identifying biomarkers associated with specific symptom domains and with prospective risk for PTSD development, enabling more efficient targeting of interventions.[10] The startle response might be such a candidate biomarker, but it is unknown whether altered startle responding represents a "trait" or "state" biomarker for PTSD.

The startle response is an operational measure of threat anticipation linked to fear circuit activation in humans and animals (e.g.,[11,12]). Heightened startle responding is a commonly endorsed symptom of PTSD that has been a long-standing criterion within the evolving versions of the DSM including DSM-5.[13,14] Empirical evidence for exaggerated startle magnitude in PTSD is mixed, however,[15,16] with a meta-analysis indicating only modest increases in baseline startle reactivity.[17] There are several potential reasons for modest associations. Startle hyperreactivity and hyporeactivity may be experienced by different subgroups of PTSD patients with distinct trauma-related pathology or trauma histories (e.g.,[18]). For example, PTSD resulting from a single trauma may be characterized by elevated startle reactivity whereas PTSD following multiple traumas is characterized by diminished physiological reactivity.[19] Furthermore, startle reactivity differences may not reflect current PTSD symptom state, but instead indicate increased risk of developing PTSD. There is circumstantial evidence for an association of startle reactivity with anxiety disorder risk: (1) offspring of anxiety disorder patients have increased startle reactivity compared to offspring of nonanxious parents[20–22] and (2) increased startle is linked to childhood trauma, a strong PTSD risk factor.[17] Two prospective studies were contradictory in supporting increased baseline startle reactivity as a PTSD risk factor,[23,24] although these studies were relatively small (n = 99 and 138) and had few subjects with a diagnosis of PTSD. Thus, it remains unclear if startle reactivity is a marker of state PTSD symptoms or a trait marker of PTSD risk.

Self-reported "increased startle" in PTSD subjects may refer to elevated probability of having a startle response under subthreshold conditions rather than simply heightened startle magnitude (e.g.,[25]). Patients may report elevated startle because the stimulus intensity needed to induce startle responding is lower, thus increasing the probability of startle across a wider range of stimuli rather than showing greater response magnitude per se. Thus, to parse out differences between startle threshold versus response magnitude, we examined startle reactivity across a range of intensities to identify overall magnitude differences and changes in the threshold to induce a response. We examined data extracted from the Marine Resiliency Study (MRS),[26] a large prospective study of active duty service members to test the hypotheses that (1) startle reactivity is associated with current PTSD symptoms and other stress-related symptoms and (2) predeployment startle reactivity predicts postdeployment symptom development. Since PTSD is a heterogeneous condition,[27,28] we examined associations of startle with overall PTSD symptoms and DSM-IV symptom clusters using a 4-factor model[29] (re-experiencing, avoidance, numbing, hyperarousal), and with general anxiety and depression symptoms.

METHODS

STUDY DESIGN AND PARTICIPANTS

MRS[26] is a longitudinal study of 2,600 U.S. Marines and Navy Corpsmen (typically treating/aiding combat wounded) around combat deployments to Iraq or Afghanistan (1-month predeployment, immediately postdeployment, and ~3- and ~6-months postdeployment). Institutional review boards of the University of California San Diego, VA San Diego Research Service, and Naval Health Research Center approved the study, and written informed consent was obtained from all participants.

Of the 2,592 participants with valid predeployment startle reactivity, 2,571 completed predeployment psychiatric measures and 1,632 completed psychiatric measures at 6-month postdeployment. To test both hypotheses, we used predeployment startle data (largest N). To predict PTSD-risk, we used predeployment startle to predict symptoms at the 6-month time point (reflecting greatest chronicity after trauma).

MEASURES

Complete MRS methods are described elsewhere[26]; only measures relevant to the present study are presented here.

STARTLE THRESHOLD TEST

Stimuli and Apparatus. Startle pulses were delivered using a San Diego Instruments (San Diego, CA, USA) SR-HRLAB EMG system as previously described.[30–32] EMG data (1-KHz sampling rate) were amplified, rectified, band-pass filtered (100–1,000 Hz), and smoothed (5-point rolling-average). All trials were reviewed by trained technicians using standard methods to remove artifact (e.g., responses that began before or 100 ms after probe onset were removed). Details are in supplementary materials.

Experimental Procedure. Prior to startle testing, hearing threshold was examined using 100, 500, 3,000, and 6,000 Hz tones at 35 dB[A] via a Grason–Stadler Audiometer (Eden Prairie, MN, USA). The startle threshold task was modeled after prior research.[25] After a 5 min acclimation, four 114-dB[A] broadband pulses were presented to assess "maximal" startle reactivity scores. Startle probes were then presented in pseudorandom order across six intensities: 80, 85, 90, 95, 100, and 105 dB[A] (5 pulses/trial-type). Probes had instantaneous rise/fall time, were 40 ms in duration, with intertrial interval average of 15 s. A 70 dB[A] broadband background noise was continuous.

ASSESSMENT OF PSYCHIATRIC SYMPTOMS

Posttraumatic Stress Disorder. Predeployment and 6-month postdeployment PTSD symptom severity was assessed using the Clinician-Administered PTSD Scale (CAPS),[33] a structured diagnostic interview designed to assess DSM-IV PTSD symptoms[34–36] with high convergent and divergent validity.[37] Interrater reliability was high between CAPS interviewers and trained observers making independent ratings, with an intraclass correlation coefficient = 0.99 ($n = 261$). CAPS was scored as zero if participants did not endorse any criterion A traumatic events according to DSM-IV on the Life Event Checklist (LEC),[38] a survey of criterion A events experienced or witnessed (0–16 range). CAPS total score (0–136 range) served as a continuous measure of PTSD symptoms. Four CAPS subscales were also calculated[29]: re-experiencing (B1-5), hyperarousal (D1-5), avoidance (C1-2), and numbing (C4-6). DSM-IV PTSD diagnostic criteria were defined as endorsing at least one criterion A event, one cluster B symptom, three cluster C symptoms, and two cluster D symptoms whereas "subthreshold" PTSD was defined as endorsing at least one criterion A event, one cluster B symptom, and either three cluster C or two cluster D symptoms.[39,40]

Anxiety. Predeployment and 6-month postdeployment anxiety symptoms were assessed with the Beck Anxiety Inventory (BAI),[41] a 21-item questionnaire (0–63 range) of general cognitive and somatic anxiety symptoms experienced within the past week with divergent and discriminant validity.[42] BAI cognitive (items 4, 5, 9, 10, 14, 16, 17) and somatic (items 1–3, 6–8, 11–13, 15, 18–21) subscales were also calculated.

Depression. Predeployment and 6-month postdeployment depressive symptoms within the past 2 weeks were assessed with the Beck Depression Inventory II (BDI-II),[43] a 21-item questionnaire (0–63 range) with strong discriminant, convergent, and content validity.[44]

Childhood Trauma. Traumatic experiences during childhood were assessed at predeployment with a modified Childhood Trauma Questionnaire (CTQ),[45] a 34-item questionnaire (25–170 range) with strong discriminant and convergent validity.[46,47]

Deployment Stress and Combat Exposure. Stressful experiences during combat and deployment were assessed at 6-months postdeployment with four scales from the Deployment Risk and Resilience Inventory-2 (DRRI-2; Postbattle Experiences, Combat Experience, Deployment Concern, Difficult Living and Working Environment), with high criterion validity and internal consistency (0.92).[48]

ANCESTRY

To control for associations of race with startle reactivity (e.g., [49]), we used a genetically derived ancestry variable as a covariate.[50] Participants were placed into four groups: Caucasian ($N = 1,588$); African-American ($N = 161$); Hispanic and Native American ($N = 459$); and Asian/Other ($N = 363$; details in supplementary materials).

STATISTICAL ANALYSIS

Analyses were conducted using statistical software package R, version 3.1.1,[51] and Statistical Package for the Social Sciences, SPSS version 21.0.0.[52] To best analyze curvilinear response differences in predeployment startle magnitude as startle stimulus intensity increased, a Latent Class Mixture Model (LCMM; R package lcmm)[53] was used. This approach enables identification of homogenous subgroups of participants within the full cohort that followed unique trajectories of startle magnitude increases across stimulus intensities. Group membership classifications were then used as an independent variable to indicate participant startle tendency across stimulus intensities. The model was constructed iteratively, with curvilinear trajectory being specified and additional groups being added until model fit either no longer improved or membership in any class dropped below 10% of the sample.

MRS measures of psychiatric symptoms (CAPS, BAI, BDI-II) at predeployment and 6-month postdeployment were positively skewed, overdispersed, and had an excess of zero scores, as previously reported.[54] Hence, zero-inflated negative binomial regression (ZINBR) was the appropriate analytic method. ZINBR uses maximum likelihood to model outcomes via two component models: logistic regression (zero model) predicting probability of a zero score, and negative binomial regression (count model) predicting total score.

Predeployment startle threshold class was included as a factor in ZINBR analyses to predict symptoms at

TABLE 1. Comparisons of characteristics and psychiatric symptoms between startle threshold classes

Predeployment characteristic	High-threshold ($n = 1,318$)	Moderate-threshold ($n = 987$)	Low-threshold ($n = 266$)	*P*-value
Age[a]	22.69 (3.62)	22.88 (3.42)	22.93 (3.20)	.33
Ancestry[b], %				**<.001**[c]
Caucasian	55.3	68.9	68.0	
African-American	8.5	4.2	2.6	
Hispanic/Native American	18.2	17.0	19.2	
Asian/Other	17.9	9.9	10.2	
Marital status[b], %				.26
Never Married	62.3	61.2	59.0	
Married	35.1	34.4	36.8	
Divorced	1.5	3.0	2.6	
Separated	1.1	1.5	1.5	
CTQ[a]	40.65 (14.15)	40.12 (13.59)	39.05 (12.59)	.22
Childhood Physical abuse	8.8 (4.1)	8.8 (4.0)	8.6 (3.7)	.76
Childhood sexual abuse	5.6 (2.2)	5.6 (2.2)	5.5 (2.0)	.87
Lifetime trauma (LEC)[a]	4.96 (3.23)	5.11 (3.26)	5.44 (3.24)	.08
Months spent in military[a]	36.28 (36.08)	35.60 (34.40)	37.54 (31.72)	.70
Months remaining in enlistment[a]	27.67 (13.31)	27.74 (13.26)	26.59 (13.87)	.43
Any previous deployment[b], %	**49.5**	**51.7**	**58.6**	**.03**[d]
Total previous deployments[a]	0.84 (1.1)	0.86 (1.1)	0.97 (1.2)	.20
Total lifetime TBI with LOC[a]	0.59 (0.99)	0.64 (0.94)	0.53 (0.85)	.18
CAPS[a]	**7.02 (12.94)**	**6.03 (10.80)**	**8.68 (13.84)**	**.005**[e]
BAI[a]	5.99 (5.71)	5.99 (5.81)	6.82 (6.15)	.09
BDI-II[a]	6.47 (7.74)	6.61 (7.77)	7.45 (7.94)	.17
PTSD diagnosis, traditional[f], %[b]	3.8	3.1	5.6	.15
PTSD diagnosis, subsyndromal[g], %[b]	**7.8**	**6.5**	**11.3**	**.03**[h]
Postdeployment characteristic	High-threshold ($n = 835$)	Moderate-threshold ($n = 632$)	Low-threshold ($n = 165$)	
DRRI-2[a]	0.1 (0.80)	−0.03 (0.81)	0.07 (0.85)	.17
Combat and posthattle experience[a]	0.30 (0.23)	0.29 (0.23)	0.32 (0.24)	.34
CAPS[a]	9.67 (16.12)	9.37 (15.57)	9.73 (14.80)	.94
BAI[a]	4.79 (7.84)	4.77 (8.09)	4.89 (7.04)	.98
BDI-II[a]	5.37 (7.20)	4.85 (6.74)	5.86 (6.56)	.11
PTSD diagnosis, traditional[f], %[b]	5.6	5.2	5.3	.95
PTSD diagnosis, subsyndromal[g], %[b]	10.4	10.6	11.8	.87

[a]One-way ANOVA analyses performed.
[b]Chi-squared test of distribution performed.
[c]Post hoc tests indicate lower proportion of high-threshold participants were Caucasian and a higher proportion were African-American and Asian/Other ($P < .001$), higher proportion of moderate-threshold participants were Caucasian and a lower proportion were African-American and Asian/Other ($P < .001$), and higher proportion of low-threshold participants were Caucasian ($P = .03$) and a lower proportion were African-American ($P = .01$).
[d]Post hoc tests indicate a higher percentage of participants in the low-threshold than high-threshold class with previous deployment experience ($P = .023$).
[e]Post hoc tests indicate lower score in moderate-threshold class than low-threshold class ($P = .005$).
[f]Traditional PTSD criteria: criterion A event, at least 1 cluster B symptom, 3 cluster C symptoms, and 2 cluster D symptoms, with minimum frequency ratings of 1 and minimum intensity ratings of 2 on CAPS.
[g]Subsyndromal PTSD criteria: criterion A event, at least 1 cluster B symptom, 3 cluster C or 2 cluster D symptoms, with minimum frequency ratings of 1 and minimum intensity ratings of 2 on CAPS.
[h]Post hoc tests indicate higher proportion of participants in the low-threshold class than moderate-threshold class met subsyndromal PTSD criteria ($P = .02$).
Significant associations are highlighted in bold.

either predeployment or 6-month postdeployment. Because the moderate-startle class displayed the lowest predeployment CAPS scores (Table 1), it was chosen as the referent group in ZINBR analyses to detect symptom increases in the other classes. Ancestry and deployment history differed between startle threshold classes (Table 1), thus these variables were included in the model. Number of correct responses on the hearing test was included to account for hearing differences potentially affecting startle reactivity. A composite of DRRI-2 scales was included to account for differences in combat and deployment experience. An interaction between

DRRI-2 and startle class was examined but it did not improve the model. Multiple other potential confounders were evaluated, including predeployment depression (via BDI-II), sleep quality, caffeine and tobacco use, and traumatic brain injury (TBI), but none improved the model.

Startle threshold class membership at predeployment was the primary predictor variable. The zero and count models were primarily used to predict responses on CAPS and CAPS subscales (re-experiencing, avoidance, numbing, hyperarousal) at both predeployment and 6-month postdeployment. Secondary ZINBR models including trauma history variables (CTQ and LEC) were conducted to examine effects of childhood and lifetime trauma burden on the relationship between startle threshold and PTSD symptoms. Additional secondary analyses predicted predeployment and 6-month postdeployment responses on BAI, BAI subscales (somatic, cognitive), and BDI-II.

ZERO MODEL: PREDICTING ABSENCE OF PSYCHIATRIC SYMPTOMS

Exponentiated coefficients of the zero model were interpreted as odds of a zero score. The zero model intercept reflects the base probability of having a zero score given that a participant was in the moderate-threshold class, Caucasian, never before deployed, with average hearing. Average DRRI-2 and PTSD symptom scores at predeployment were also referents when predicting 6-month postdeployment scores.

COUNT MODEL: PREDICTING TOTAL PSYCHIATRIC SYMPTOMS

Exponentiated coefficients of the count model represent multiplicative change in predicted measure score per unit change in a given predictor. The count model intercept reflects a predicted symptom score given the same referents as described for the zero model.

RESULTS

STARTLE THRESHOLD CLASS

The LCMM showed three distinct classes of growth across stimulus intensity levels (Fig. 1). The high-threshold class (51.3% of participants) was characterized by relatively flat trajectory, only rising in magnitude at the highest dB[A] levels. The moderate-threshold class (38.4% of participants) was characterized by a slope of increasing startle magnitude across dB[A] levels. The low-threshold class (10.3% of participants) was characterized by an abruptly steep slope, distinguishable even at low dB[A] levels.

SAMPLE CHARACTERISTICS BY STARTLE THRESHOLD CLASS

Overviews of pre- and postdeployment MRS cohort characteristics have been reported previously.[25,45] Predeployment demographic and descriptive data are presented for each startle threshold class (Table 1). Chi-squared tests indicated significant predeployment differences between startle threshold classes in racial ancestry (χ^2 (6, n = 2,592) = 72.95; $P < .001$). More participants in the low-threshold class had been previously deployed compared to other classes (χ^2 (2, n = 2,585) = 6.96; $P = .03$). Startle threshold classes did not differ at predeployment in age, marital status, total number of prior deployments, total months spent in the military, total months remaining in military enlistment, total lifetime TBI with loss of consciousness, or childhood trauma measures. The low-threshold class tended to have more lifetime trauma ($P < .08$).

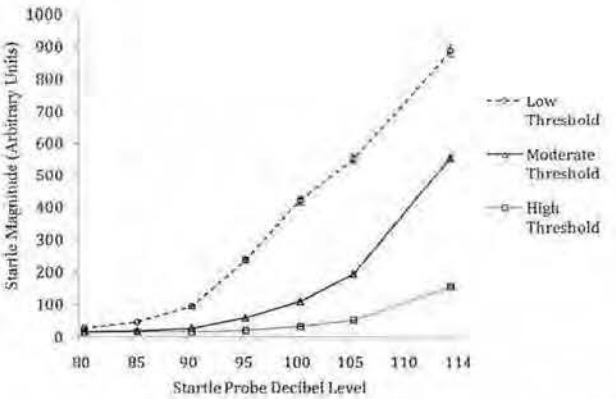

Figure 1. Mean startle threshold class response across decibel level, ±1 SEM. Startle class trajectories identified using Latent Class Mixture Model.

Pre- and postdeployment measures of psychiatric symptoms are presented for each threshold class (Table 1). One-way analysis of variance (ANOVA) indicated significant threshold class differences in CAPS at predeployment (F (2, 2,586) = 5.31; $P = .005$) but not postdeployment. Deployment trauma did not differ across classes. Although classes did not differ in the percent meeting DSM-IV PTSD diagnostic criteria at predeployment (3.7% of participants), significantly more participants met subthreshold PTSD in the low-threshold compared to moderate-threshold class (χ^2 (2, n = 2,592) = 6.84; $P = .03$). There were no class differences in full or subthreshold PTSD at postdeployment. Threshold classes did not differ on BAI or BDI-II.

ZERO-INFLATED NEGATIVE BINOMIAL REGRESSION

For clarity, we have only depicted ZINBR results for threshold class as a predictor of PTSD at predeployment (Table 2) and postdeployment (Table 3) in the body of the paper. Full models with all predictors are included as supplementary materials.

RELATIONSHIP BETWEEN STARTLE THRESHOLD AND CURRENT PTSD SYMPTOMS

Count Model. In participants endorsing PTSD symptoms, high-threshold class membership increased predeployment predicted CAPS score by a factor of 1.14 (14%; $P = .04$), CAPS-reexperiencing by a factor of

TABLE 2. Zero-inflated negative binomial regression predicting predeployment CAPS total score and subscales

Outcome measure	Model	Variable[a]	Estimate (SE)	*P*-value	Predicted measure total[b,c]	Ratio (95% CI)[d]
CAPS total	Count	(Intercept)	2.90 (0.21)	<.001	9.96	(8.07–12.29)
		High-threshold*	**0.14 (0.06)**	**.04**		**1.14 (1.01–1.29)**
		Low-threshold	0.17 (0.10)	.08		1.18 (0.98–1.43)
	Zero	(Intercept)	−0.63 (0.35)	.07	53.05%	(44.32–61.59%)
		High-threshold	0.03 (0.09)	.76		1.02 (0.86–1.23)
		Low-threshold*	**−0.38 (0.15)**	**.01**		**0.68 (0.50–0.92)**
CAPS-reexperiencing	Count	(Intercept)	2.17 (0.21)	<.001	5.17	(4.19–6.38)
		High-threshold*	**0.18 (0.07)**	**.01**		**1.20 (1.05–1.36)**
		Low-threshold	0.14 (0.11)	.17		1.15 (0.59–2.26)
	Zero	(Intercept)	0.14 (0.34)	.68	64.36%	(56.25–71.73%)
		High-threshold	0.12 (0.10)	.22		1.13 (0.93–1.36)
		Low-threshold	0.02 (0.15)	.88		1.02 (0.76–1.39)
CAPS-avoidance	Count	(Intercept)	1.36 (0.24)	<.001	4.20	(3.30–5.34)
		High-threshold*	**0.16 (0.07)**	**.02**		**1.17 (1.02–1.35)**
		Low-threshold	0.10 (0.11)	.38		1.10 (0.89–1.36)
	Zero	(Intercept)	0.74 (0.39)	.06	79.28%	(72.14–84.96%)
		High-threshold	0.06 (0.11)	.57		1.06 (0.86–1.32)
		Low-threshold	−0.11 (0.17)	.52		0.89 (0.63–1.26)
CAPS-numbing	Count	(Intercept)	1.87 (0.22)	<.001	6.02	(4.83–7.50)
		High-threshold*	**0.21 (0.08)**	**.01**		**1.24 (1.06–1.44)**
		Low-threshold	0.08 (0.11)	.46		1.08 (0.88–1.33)
	Zero	(Intercept)	0.89 (0.40)	.03	89.74%	(85.43–92.88%)
		High-threshold	0.06 (0.13)	.60		1.06 (0.83–1.36)
		Low-threshold*	**−0.58 (0.18)**	**<.001**		**0.56 (0.40–0.79)**
CAPS-hyperarousal	Count	(Intercept)	2.29 (0.16)	<.001	7.31	(6.23–8.58)
		High-threshold	0.02 (0.05)	.73		1.02 (0.91–1.13)
		Low-threshold	−0.03 (0.08)	.72		0.97 (0.84–1.13)
	Zero	(Intercept)	0.33 (0.34)	.33	79.94%	(73.93–84.85%)
		High-threshold	−0.12 (0.19)	.21		0.88 (0.73–1.07)
		Low-threshold*	**−0.57 (0.15)**	**<.001**		**0.57 (0.42–0.76)**

[a]Moderate-threshold membership used as referent group for high-threshold and low-threshold class membership.
[b]Estimate for participant who is Caucasian, never before deployed, with average hearing.
[c]For the zero model, base probability of a predicted score of 0.
[d]95% confidence interval for predictor coefficient. Count model coefficients indicate multiplicative change in predicted measure score per unit change in predictor. Zero model coefficients indicate predicted factor change in odds of a zero score for measure per unit change in predictor.
*Predictor P-value <.05
Significant associations are highlighted in bold.

1.20 (20%; $P = .01$), CAPS-avoidance by a factor of 1.17 (17%; $P = .02$), and CAPS-numbing by a factor of 1.24 (24%; $P = .003$), but was not associated with CAPS-hyperarousal. Low-threshold class membership did not significantly predict CAPS.

Zero Model. High-threshold class membership did not significantly affect predeployment odds of a zero score on CAPS or CAPS-subscales. Low-threshold class membership decreased predeployment odds of a zero score on CAPS by a factor of 0.68 (32%; $P = .01$), CAPS-numbing by a factor of 0.56 (44%; $P < .001$), and CAPS-hyperarousal by a factor of 0.57 (43%; $P < .001$), but did not affect odds of zero scores on CAPS-reexperiencing or CAPS-avoidance.

RELATIONSHIP BETWEEN STARTLE THRESHOLD AND FUTURE PTSD RISK

Neither high-threshold nor low-threshold class membership at predeployment were significantly associated with postdeployment CAPS in the count or zero models.

Secondary Analyses. For full results of secondary models see supplementary materials. When trauma-burden measures were included in ZINBR models, associations between low-threshold class and PTSD symptoms remained significant (Ps = <.001–.01). High-threshold class association with CAPS-avoidance also remained significant ($P = .04$) whereas associations with CAPS-total, CAPS-reexperiencing and CAPS-numbing did not. Removing participants that denied ever experiencing a category A event ($N = 80$, 3%) from the analyses did not change the findings (data not shown). For predicting anxiety and depression, low-threshold class membership decreased the odds of a zero score for BAI-somatic by a factor of 0.64 (36%; $P < .04$) whereas class membership was not associated with BDI-II. To examine if trauma burden is related to startle threshold among individuals with PTSD, we examined class differences in CTQ and LEC among PTSD cases. Individuals meeting predeployment diagnosis for PTSD endorsed more childhood trauma

TABLE 3. Zero-inflated negative binomial regression predicting 6-months postdeployment CAPS total score and subscales

Outcome measure	Model	Variable[a]	Estimate (SE)	*P*-value	Predicted measure total[b,c]	Ratio (95% CI)[d]
CAPS total	Count	(Intercept)	2.56 (0.26)	<0.001	12.00	(9.25–15.56)
		High-threshold	0.04 (0.07)	0.53		1.04 (0.91–1.20)
		Low-threshold	−0.05 (0.11)	0.66		0.95 (0.767–1.18)
	Zero	(Intercept)	0.80 (0.48)	0.10	43.71%	(32.46–55.66%)
		High-threshold	−0.03 (0.14)	0.78		0.97 (0.75–1.24)
		Low-threshold	−0.12 (0.22)	0.59		0.89 (0.58–1.35)
CAPS-reexperiencing	Count	(Intercept)	1.81 (0.29)	<0.001	5.67	(4.24–7.57)
		High-threshold	0.04 (0.08)	0.56		1.04 (0.90–1.21)
		Low-threshold	−0.12 (0.12)	0.29		0.88 (0.70–1.11)
	Zero	(Intercept)	1.46 (0.48)	0.002	68.64%	(57.52–77.96%)
		High-threshold	−0.06 (0.13)	0.63		0.94 (0.73–1.21)
		Low-threshold	−0.38 (0.21)	0.07		0.68 (0.45–1.03)
CAPS-avoidance	Count	(Intercept)	1.72 (0.30)	<0.001	4.46	(3.30–6.02)
		High-threshold	0.04 (0.08)	0.63		1.04 (0.88–1.23)
		Low-threshold	−0.09 (0.13)	0.49		0.92 (0.71–1.18)
	Zero	(Intercept)	2.08 (0.52)	<0.001	86.46%	(79.16–91.49%)
		High-threshold	−0.21 (0.15)	0.15		0.81 (0.61–1.08)
		Low-threshold	−0.37 (0.23)	0.10		0.69 (0.44–1.08)
CAPS-numbing	Count	(Intercept)	2.09 (0.36)	<0.001	6.45	(4.50–9.25)
		High-threshold	0.15 (0.10)	0.13		1.16 (0.96–1.40)
		Low-threshold	0.01 (0.15)	0.96		1.01 (0.75–1.35)
	Zero	(Intercept)	3.36 (0.63)	<0.001	89.06%	(81.02–93.77%)
		High-threshold	−0.14 (0.15)	0.37		0.87 (0.64–1.18)
		Low-threshold	0.00 (0.25)	0.99		1.00 (0.61–1.65)
CAPS-hyperarousal	Count	(Intercept)	2.26 (0.20)	<0.001	8.24	(6.75–10.07)
		High-threshold	0.02 (0.06)	0.76		1.02 (0.91–1.14)
		Low-threshold	−0.05 (0.09)	0.61		0.96 (0.80–1.14)
	Zero	(Intercept)	0.92 (0.47)	0.05	59.71%	(48.09–70.34%)
		High-threshold	0.17 (0.12)	0.17		1.18 (0.93–1.51)
		Low-threshold	0.02 (0.20)	0.94		1.02 (0.68–1.51)

[a]Moderate-threshold membership used as referent group for High-threshold and Low-threshold class membership.
[b]Estimate for participant who is Caucasian, never before deployed, with average hearing and DRRI, and with zero scores on measures at predeployment.
[c]For the zero model, base probability of a predicted score of 0.
[d]95% confidence interval for predictor coefficient. Count model coefficients indicate multiplicative change in predicted measure score per unit change in predictor. Zero model coefficients indicate predicted factor change in odds of a zero score for measure per unit change in predictor.

($P < .001$) and physical abuse ($P = .001$) in the high-threshold class, but there were no class differences for LEC.

DISCUSSION

This study examined if differences in startle threshold are associated with PTSD symptom severity (PTSD state) and/or are predictive of trait risk for developing PTSD after deployment. Startle responses were fitted into three distinct growth classes across stimulus intensity levels, with classes defined by high, moderate, and low thresholds. ZINBR models indicated that relative to moderate-threshold, high-threshold class membership at predeployment was associated with more severe predeployment symptoms on CAPS-total, CAPS-reexperiencing, CAPS-avoidance, and CAPS-numbing. Relative to moderate-threshold, low-threshold class membership was associated with decreased likelihood of being symptom-free at predeployment on CAPS-total, CAPS-numbing, CAPS-hyperarousal, and BAI-somatic. These findings suggest that low startle threshold may be associated with increased likelihood of endorsing current PTSD and anxiety symptoms, whereas high-threshold responding is associated with increased PTSD severity once symptoms emerge. Previous research supports that "baseline" EMG startle reactivity is associated with PTSD symptom state that can remit after treatment,[55] although there are some inconsistencies likely due to methodological differences.[56] Predeployment startle threshold class did not predict postdeployment psychiatric symptoms, suggesting that startle threshold does not represent a trait risk-factor for developing PTSD or anxiety. The large cohort size and pre- and postdeployment assessments used here build on previous prospective research finding that startle sensitization develops along with PTSD symptoms rather than representing a preexisting risk factor.[57] PTSD risk has been asso-

ciated, however, with startle in response to conditioned fear-cues or aversive stimuli,[24] suggesting that EMG responses during threat may probe different mechanisms of PTSD risk than "baseline" startle tasks. Neither current depression nor development of depression symptoms were predicted by threshold class, consistent with previous findings that altered startle response is associated with fear and anxiety but not depression.[58–60]

The association of current PTSD symptoms with both low and high startle thresholds is consistent with findings that many PTSD patients show exaggerated startle reactivity similar to other fear-based disorders, whereas PTSD patients with particularly severe trauma histories demonstrate blunted startle similar to disorders of pervasive apprehension and negative affect.[18,19] Our finding that low-threshold responding was associated with PTSD and somatic anxiety symptoms is consistent with a fear-based PTSD presentation. That high-threshold reactivity was associated with more severe PTSD symptoms but not anxiety symptoms is consistent with the idea that a subset of PTSD patients show diminished defensive responding. In secondary analyses, low-threshold startle was associated with CAPS-total, -numbing, and -hyperarousal symptoms above and beyond variance accounted for by childhood and lifetime trauma, suggesting that elevated startle reactivity develops independently from trauma exposure. Alternatively, when accounting for trauma burden high-threshold startle only remained predictive for avoidance, but not CAPS-total or re-experiencing symptoms. There were no differences between threshold classes on measures of lifetime trauma burden or depression, although among the 96 individuals who met DSM-IV criteria for PTSD at predeployment, those with high startle threshold had greater history of childhood trauma and physical abuse. Together, these results suggest that PTSD following high childhood and lifetime trauma burden may be characterized by diminished physiological reactivity, with trauma burden accounting for much of the association between blunted startle and PTSD severity, whereas elevated startle may be associated with PTSD symptoms independent from trauma history.

These findings suggest that a moderate startle-threshold may indicate minimal current PTSD symptomatology relative to high or low startle thresholds, and may have important implications regarding the relationship between PTSD and abnormalities in startle response neurocircuitry. Startle reactivity is modulated by the amygdala and bed nucleus of the stria terminalis, via projections to nodes of the primary startle circuit in the brainstem that mediate startle.[61] Exaggerated startle reactivity is putatively related to amygdala hyperactivity in PTSD (e.g., [62]), but several different neurobiological processes might contribute to low startle being associated with increased psychiatric symptoms. During severe stress, the periaqueductal gray (PAG) inhibits startle in favor of other defensive behaviors resulting in an inverted-U shaped dose-response function between stressor intensity and startle response.[63,64] Signaling pathways linked to inverted U-shaped effects on startle reactivity that are abnormal in PTSD include corticotropin releasing factor (CRF) and glucocorticoid signaling. PTSD patients exhibit increased CRF levels in cerebrospinal fluid[65–68] and increased glucocorticoid sensitivity.[69] Moderate CRF and glucocorticoid levels induce increased startle whereas high doses induce reduced startle reactivity[70–73] CRF-induced inhibition or potentiation of startle also depend on neural sources of CRF hypersignaling.[74] Future research is needed to determine if these neural circuits and signaling pathways are linked to different startle thresholds. Understanding the neurobiological mechanisms influencing startle threshold might help identify separate functional pathologies across PTSD and other anxiety disorders.

This study has important limitations. First, this cohort was entirely male so it is unknown if the findings are applicable to females, particularly given recent gender differences found in the relationship between startle reactivity and PTSD.[75] Second, this cohort was young, generally healthy, and highly screened, all of which may limit generalizability. Third, the types of traumas faced by this military cohort may differ from traumas experienced by civilians. Fourth, participants developing symptomatology postdeployment may have been less likely to remain in the military until postdeployment assessment. Few study participants met PTSD diagnostic criteria at pre- or postdeployment, thus this study may have been underpowered to detect the relationship between startle threshold and severe PTSD symptoms.

Overall, these findings indicate that distinct patterns of startle reactivity across high and subthreshold stimulus intensity are associated with current PTSD symptom "state," but not with trait risk for developing psychiatric symptoms. Moderate startle-threshold was associated with fewer current PTSD symptoms relative to low- and high-thresholds. Future research should investigate the relationship between lifetime trauma burden and PTSD symptom severity with blunted startle responding. Additionally, future research should examine the biological underpinnings of startle threshold as an intermediate phenotype for PTSD state. Improved understanding of startle and other PTSD-related biomarkers may facilitate targeting of treatment and prevention strategies.

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On the Road to Translation for PTSD Treatment: Theoretical and Practical Considerations of the Use of Human Models of Conditioned Fear for Drug Development

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Abstract The use of quantitative, laboratory-based measures of threat in humans for proof-of-concept studies and target development for novel drug discovery has grown tremendously in the last 2 decades. In particular, in the field of posttraumatic stress disorder (PTSD), human models of fear conditioning have been critical in shaping our theoretical understanding of fear processes and importantly, validating findings from animal models of the neural substrates and signaling pathways required for these complex processes. Here, we will review the use of laboratory-based measures of fear processes in humans including cued and contextual conditioning, generalization, extinction, reconsolidation, and reinstatement to develop novel drug treatments for PTSD. We will primarily focus on recent advances in using behavioral and physiological measures of fear, discussing their sensitivity as biobehavioral markers of PTSD symptoms, their response to known and novel PTSD treatments, and in the case of d-cycloserine, how well these findings have translated to outcomes in clinical trials. We will highlight some gaps in the literature and needs for future research, discuss benefits and limitations of these outcome measures in designing proof-of-concept trials, and offer practical guidelines on design and interpretation when using these fear models for drug discovery.

Keywords Posttraumatic stress disorder · Fear · Anxiety · Panic disorder · D-cycloserine · Extinction · Exposure · Consolidation · Norpepinephrine

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Contents

1 Introduction

1.1 Posttraumatic Stress Disorder Prevalence and Treatment Options

Posttraumatic stress disorder (PTSD) affects 7–8 % of the general US population and is higher in recently deployed combat veterans (up to 20 %) (Thomas et al. 2010). Mental disorders, in particular PTSD, are associated with higher rates of physical symptoms, chronic physical illness, and overall mortality (for review see Baker et al. 2009). Research shows that this increased liability of physical disease translates into greater non-mental health medical service utilization (e.g., O'Donnell et al. 2013), creating substantial burdens for the patients, families, and societal resources. Best evidence treatment for PTSD includes cognitive behavioral therapies, i.e., cognitive processing therapy (CPT) and prolonged exposure (PE), and psychotropic medications (Institute of Medicine 2014). Although cognitive behavioral approaches have proven efficacy for PTSD, non-response can be as high as 50 %, leaving unresponsive or partially responsive patients with PTSD reliant upon pharmacotherapy (Baker et al. 2009; Institute of Medicine 2014; Berger et al. 2009). As with many psychiatric disorders, the pharmacological tool kit for PTSD treatment is relatively small, predominantly selective serotonin or norepinephrine reuptake inhibitors (SSRI/SNRI) and adjunctive treatments such as prazosin, a sympatholytic drug with alpha-1 receptor blocking activity (Baker et al. 2009; Steckler and Risbrough 2012). These medications also have high non-response rates as well as side effects (Baker et al. 2009; Steckler and Risbrough 2012). There is an unquestionable need to advance development of new treatments for PTSD, with part of this effort lying in developing innovative approaches to drug development in clinical populations.

One of the difficulties of identifying biological mechanisms for PTSD, and thus in turn developing beneficial treatments, is the heterogeneous patient population and wide spectrum of potential symptoms. According to the DSM-5 (American Psychiatric Association 2013), PTSD now comprises 20 individual symptoms. These symptoms are grouped into four symptom clusters: persistent intrusive memories of the trauma, hyperarousal and reactivity, avoidance of stimuli related to the trauma event, and negative alterations in cognitions and mood. Thus, there is a wide range of symptoms that can be endorsed to comprise a PTSD diagnosis, with many possible patterns of symptom type and severity across these clusters (Galatzer-Levy and Bryant 2013). This heterogeneity suggests that several potential biological mechanisms could drive the development of PTSD. This multiplicity of potential biological mechanisms will induce substantial variance in how any given treatment will affect a patient's treatment response.

As such, the potential for numerous different underlying pathologies in patient groups makes identification of specific mechanisms across the population very difficult. One approach to this problem is to identify biological or behavioral phenotypes that are highly represented in the diagnostic class compared to specific symptoms so as to target a "core" biological pathway that is disrupted in most patients. This approach assumes that the heterogeneity is due to noise in the self-report measurements of symptoms and how they are experienced and/or articulated, but perhaps only a few biological mechanisms actually drive clinical dysfunction. The second potential approach is to identify phenotypes that are relevant to particular symptom classes that are most severe in a given individual. This approach assumes that certain discrete phenotypes may better classify dimensions of specific symptoms experienced by subpopulations within the diagnostic group as a whole, each with potentially differing biological mechanisms (Schmidt 2015).

Development of laboratory-based behavioral measures of disease-related processes is a critical component of the evolution of translational research (Bowers and Ressler 2015). These tasks can bridge complex clinical presentations with discrete biological mechanisms (Braff 2015; Gottesman and Gould 2003; Rasetti and Weinberger 2011; Risbrough 2010). This strategy is now endorsed by the Research Domain Criteria (RDoC) project by the National Institute for Mental Health (Cuthbert and Insel 2013). Similarly, industry and academia have now increasingly turned to biological and behavioral markers in initial proof-of-concept studies to identify efficacy across specific emotional and cognitive constructs of PTSD to guide future phase II clinical trial designs. Here, we will discuss the promise and pitfalls of commonly used laboratory-based measures of conditioned fear processes to support novel drug development for PTSD.

1.2 *Considerations of Benefits and Limitations of Laboratory-Based Measures of Behavior for Drug Discovery*

1.2.1 Benefits of Validated Behavioral Phenotypes to Complement Symptom Assessments

(1) Objective, quantifiable assessments of function compared to self-report.
(2) Often have well characterized biological mechanism(s) and neural circuit(s).
(3) Responses are predictably controlled by specific experimental parameters in keeping with their use as an operational measure of a defined construct (e.g., anxiety, fear, arousal).
(4) Observable behaviors enable cross-species translation to lower order organisms for direct mechanistic studies and drug development (Donaldson and Hen 2015).
(5) Compared to symptoms, laboratory-based measures are observable across healthy controls and clinical populations, supporting efforts to disentangle mechanisms that cause risk versus mechanisms related to symptom onset and severity. This point is particularly important for informing treatment approaches, e.g., prophylactic versus therapeutically.
(6) Unlike symptoms, behaviors can be measured in unaffected relatives to aid in identification of genetic risk factors [e.g., behavioral endophenotypes or intermediate phenotypes (Lenzenweger 2013)].
(7) Because they are typically based on continuous measures, they offer more statistical power than dichotomous diagnostic classes.
(8) Most importantly for drug discovery, they may probe a more specific conceptual target for pharmacotherapy indicated by preclinical studies (e.g., effective for enhancing fear extinction). This last point is the primary reason behavioral tests are being used more frequently, as they may offer a greater ability to translate drug effects that are based on specific circuit actions and behavioral effects in preclinical models.

1.2.2 Limitations

(1) Lack of specificity: It is often the case that some individuals with disrupted performance in a behavioral task may not show overt functional deficits or clinical presentation. For example, menstrual cycle phases are associated with reductions in fear extinction in healthy women (Glover et al. 2015; Milad et al. 2006).
(2) In the context of genetic studies, even relatively "simple" or discrete laboratory-based behaviors do not guarantee greater heritability or simpler genetic architecture than the disorder (Greenwood et al. 2007), as would be

hoped from an intermediate phenotype or endophenotype. For example, even a behavior as simple as the startle reflex may be modulated by a huge array of biological pathways (Zhang et al. 2011).

(3) Behaviors that initially seemed relatively simple in terms of core neural circuit, e.g., extinction requiring prefrontal cortex activation of inhibitory circuits in the amygdala, can have extensive modulatory circuits that may play a stronger role in how this phenotype is altered in a given disorder compared to the "core neural circuit" (Acheson et al. 2015c; Maren and Holmes 2015; Milad et al. 2013). Thus, using behavioral performance as a proxy for the function of a specific neural circuit or brain region is limited unless it is accompanied by other information such as functional imaging.

Here, we will review the state of the art in laboratory-based measures of fear response in assessing symptom state and response to treatment in healthy controls and PTSD patients within the fear learning domains. We will also offer some practical considerations for study design and interpretation pitfalls for future planning of drug efficacy using these measures.

2 Learned Fear Processes

One of the predominant features of PTSD symptoms is robust, uncontrollable memories of the traumatic event, i.e., re-experiencing. Secondly, external or internal cues that act as trauma reminders induce re-experiencing with flashbacks and dissociation at the most severe end of the spectrum, as well as strong emotional and physiological fear responses including intense anxiety and panic. Unsurprisingly, the disorder is associated with implicit and explicit strategies for cue avoidance, which can be disruptive to daily function and interfere with long-term recovery. Thus, PTSD may be caused at least in part by disruption in one or more elements of the learned fear process (Lissek and van Meurs 2014). Here, we will describe common laboratory-based measures of these processes, their relationship to symptom clusters and predictive validity for subsequent clinical trials if available, response to pharmacological treatment in both controls and PTSD patients, and considerations of their use in drug development studies.

2.1 Fear Conditioning and Cued Recall

Laboratory-based tasks to elicit Pavlovian fear conditioning in humans induce learned fear typically by presenting a visual conditioned stimuli (CS), such as simple shapes or images in combination with an aversive unconditioned stimulus (US) such as shock to the wrist or air puff to the throat. Operational measurement of fear responding to the CS+ (CS associated with US) is derived by comparing

behavior or physiological responses to the CS+ compared to CSs that are not presented with the US (i.e., safety signal, CS−) or when no cues are presented. Variations include examining responses to "contextual" versus discrete CS+ [to examine phasic versus sustained fear responses (Garfinkel et al. 2014; Glenn et al. 2014; Grillon et al. 2006)].

2.1.1 Do PTSD Patients Exhibit Increased Fear Learning/Expression? Is Fear Learning/Expression Related to Specific Symptom Clusters?

The short answer is it depends on the measure. PTSD patients exhibit increased potentiated startle responses to discrete fear cues (Briscione et al. 2014; Norrholm et al. 2011) and contextual fear cues (Grillon et al. 2009b); however, increased fear is not consistently detected using other behavioral or physiological measures such as self-report or skin conductance response (SCR) (Glover et al. 2011; Milad et al. 2008). This difference may be related to specific fear circuitry that is being probed by these behavioral measures, as startle reactivity is thought to reflect "automatic" fear conditioning processes that do not rely on contingency awareness, while SCR and self-report reflect fear processes that require contingency awareness (Jovanovic et al. 2006; Tabbert et al. 2006). Given that increased startle reactivity is commonly described by patients (DSM-IV, DSM-5), startle measures of fear may specifically probe abnormal circuits and mechanisms in PTSD that drive "automatic" fear responses (Grillon 2009). As might be expected, increased fear-potentiated startle is associated with high levels of re-experiencing symptoms in PTSD patients (Norrholm et al. 2011) and attentional bias to threat (Fani et al. 2012). However, in a study that directly compared fear acquisition across subjects with PTSD, general anxiety, or depression symptoms, increased fear expression was significantly higher in individuals with general cognitive and somatic anxiety symptoms rather than PTSD or depression symptoms (Acheson et al. 2015b). Greater conditioned fear expression has also been reported in other anxiety disorders, such as panic disorder (Grillon et al. 2008) and bipolar disorder (Acheson et al. 2015c). Thus, increased fear expression may reflect a biological abnormality in subpopulations of anxiety and mood disorder patients, crossing diagnostic classifications.

2.1.2 Is Conditioned Fear Responding Sensitive to Drugs that Are Effective for PTSD?

A reasonable question when considering a laboratory-based task for drug discovery is whether the task shows predictive validity for known therapeutic compounds. Unfortunately, there is disappointingly little work in this area. In healthy controls, fear-potentiated startle responses to cues with moderate contingency prediction which are thought to elicit sustained anxiety are attenuated by sub-chronic (2 week) SSRI treatment and acute benzodiazepine treatment, while cues with 100 %

contingency for the aversive US are not (Acheson et al. 2012b; Grillon et al. 2006, 2009a). Fear conditioning as assessed by skin conductance is unaffected by sub-chronic SSRI treatment (Bui et al. 2013). These data suggest that fear-potentiated startle has predictive validity as a laboratory-based measure of fear acquisition/expression for PTSD under certain conditions, particularly when cues elicit more prolonged anxiety-like responses which may be activating differential neural circuits [e.g., bed nucleus stria terminalis, for review see Avery et al. (2015) and Burghardt and Bauer (2013)]. Does this mean discrete fear conditioning tasks are not predictive for PTSD therapeutics? Perhaps, but an alternative explanation is that current treatments, which work in 50 % or less of the population (Berger et al. 2009), are unable to treat this particular facet of the disorder and thus are not useful positive controls. Further evidence for predictive validity for SSRI effects in patients is that acute SSRI treatment potentiates fear expression in conditioned fear models, similar to accounts of increased anxiety symptoms in patients in the initial phase of SSRI treatment (Garcia-Leal et al. 2010; Grillon et al. 2007; Silva et al. 2001). Effects of prazosin, used for treating nightmares in PTSD patients and which has some efficacy in animal models of conditioned fear responding, have not been studied yet in these human models (Do Monte et al. 2013; Raskind et al. 2013). This lack of data is partly due to the requirement for incremental dosing increases over weeks to reach therapeutic levels necessary for efficacy for treatment of nightmares in PTSD, reducing the feasibility of using this compound for validation studies. Effects of behavioral therapy on conditioned fear are also relatively untested. One small study found no significant reductions in potentiated startle to trauma-related cues after exposure therapy despite >50 % reduction in symptoms (Robison-Andrew et al. 2014); however, another larger study did find that exposure therapy reduced trauma-potentiated startle (Rothbaum et al. 2014). Overall, the evidence for predictive validity in terms of sensitivity to SSRI treatment is suggestive, but there are clear nuances to the parameters and dosing strategy that must be considered if these models are to be used.

2.1.3 Does Fear Conditioning Predict Treatment Response?

Again, there is very little work in this area. One small pilot study ($n = 9 - 10$/group) showed that only patients that show discrimination in SCRs between the CS+ and CS− respond to SSRI treatment (Aikins et al. 2011). These data support the speculation that cue discrimination may probe neural circuits that are responsive to SSRI treatment, but more research is needed to confirm this preliminary finding.

2.1.4 Is There Evidence for Fear Conditioning to Be an "Intermediate Phenotype" Associated with Genes that Confer Risk for PTSD?

There is some suggestion that genes that confer risk for PTSD are also associated either with heightened fear conditioning or with disruption in ability to inhibit

conditioned fear in humans [see next section below and see Skelton et al. (2012) for review of genetic approaches to fear learning phenotypes]. Examples are genes involved in noradrenergic (ADRA2B), serotonergic (SLC6A4), and catecholamine signaling (COMT), in cellular signaling pathways that support neural plasticity [PRKCA and WWC1; for review see Wilker et al. (2014)], and in genes involved in the neuroendocrine stress response [PACAP/PAC1, Ressler et al. (2011)] and opioid signaling (Andero et al. 2013). Thus far, however, only candidate gene studies have been conducted on fear acquisition and expression phenotypes, no genome-wide association studies have been published yet.

2.2 *Fear Extinction, Reconsolidation, and Reinstatement*

Fear conditioning is vital for survival, enabling threat prediction and consequent behavioral responses to avoid harm. As cues become less predictive of aversive stimuli, however, organisms adapt to this change with reduced conditioned responding termed extinction. The process of fear extinction is subserved by a hippocampal–amygdala–prefrontal cortex circuit, with the prefrontal cortex activation of inhibitory circuits in the amygdala resulting in reduced fear responses to previously learned fear cues (for review see Milad and Quirk 2012). Extinction does not modify or "erase" the original CS–US association, but instead represents new inhibitory learning that actively competes with the original excitatory CS–US associative memory (Bouton 1993). This hypothesis is supported by a number of return of fear phenomena including reinstatement of conditioned fear, in which following fear extinction a brief re-exposure to an unpaired US induces full recovery of the original conditioned fear response (Haaker et al. 2014; Myers and Davis 2002). Modification of the original fear memory can occur, however, via reconsolidation, a period in which a memory is activated and is thus transiently labile, thought to subserve an "updating" function [see following sections below for further details (Nader 2015)].

2.2.1 Do PTSD Patients Exhibit Changes in Fear Extinction Processes?

PTSD has been described as a disorder characterized by a failure in extinction. Most trauma survivors exhibit PTSD symptoms initially after the traumatic experience; however, over time most survivors (80–90 %) will return to normal functioning, while a small subset continues to exhibit robust, debilitating trauma memories that interfere with normal functioning (Rothbaum et al. 1992; Rothbaum and Davis 2003). Extinction is a critical component to the efficacy of exposure therapy for PTSD, which exposes the patient to trauma-related memories and/or cues both in the clinic and in vivo (Craske et al. 2014).

PTSD patients exhibit reduced fear extinction learning and retention in the laboratory, indicating that poor extinction of fear responses to trauma-related cues may be a mechanism underlying PTSD (Acheson et al. 2015b; Milad et al. 2008; Norrholm et al. 2011). In a recent comparative study across subjects reporting primarily PTSD, general anxiety, or depression symptoms, extinction deficits were only observed in subjects with PTSD (Acheson et al. 2015b), suggesting that poor extinction is specifically related to trauma-related symptoms as opposed to general symptoms of low mood or ruminative anxiety. PTSD patients also exhibit functional and structural abnormalities in the fear extinction network including the hippocampus, amygdala, and frontal cortex [for review see Acheson et al. (2012a), Shvil et al. (2013)]. During extinction learning, PTSD is associated with reduced activation of the ventral medial prefrontal cortex and increased activation of the amygdala and dorsal anterior cingulate, suggesting reduced inhibitory modulation by cortical inputs to fear circuits (Shvil et al. 2013). Twin studies suggest that poor extinction observed in PTSD is associated with symptom state, rather than a vulnerability trait for PTSD (but see Lommen et al. 2013; Milad et al. 2008), suggesting it could play a role in maintenance of PTSD symptoms once they emerge. Hence, pharmacological enhancement of the neuroplasticity of this circuit is of particular interest for novel therapeutic approaches to PTSD, particularly in conjunction with exposure therapy.

2.2.2 Pharmacological Approaches for Fear Extinction in PTSD

There has been an explosion of basic and clinical research on mechanisms of fear extinction, with a large literature on the cell signaling mechanisms that mediate and modulate fear extinction learning and recall. This literature has recently been comprehensively reviewed (Maren and Holmes 2015; Singewald et al. 2015); thus, here, we will focus on a brief synopsis of the use of d-cycloserine (DCS), as this treatment is the most advanced, providing a primer in the successes and difficulties of translating animal and preclinical findings in fear behavior to clinical treatment strategies.

The concept of developing adjunctive pharmacotherapies for cognitive or exposure-based therapies was largely driven by the work of Michael Davis and Kerry Ressler. They first showed that DCS, a partial NMDA receptor agonist, administered during extinction training resulted in enhanced fear extinction recall in animals. Subsequently, they showed that DCS administered during virtual reality-based exposure therapy for fear of heights significantly increased the therapy's efficacy in reducing phobia symptoms (Ressler et al. 2004; Walker et al. 2002). These seminal papers more than a decade ago led to a burst of activity across a number of disorders, showing initial increased efficacy of DCS treatment for exposure therapies for phobias, panic disorder, and obsessive compulsive disorder which has been confirmed by two meta-analyses (Bontempo et al. 2012; Norberg et al. 2008). "High-throughput" clinical trials have been developed to test efficacy of drugs for enhancement of exposure-based therapy (Rodebaugh and Lenze 2013; Rodebaugh et al. 2013). However, the translation to exposure therapy effects in

PTSD patients is less compelling. Four studies have examined DCS enhancement of exposure therapy, with either positive effects (Difede et al. 2014), equivocal, or marginal effects (de Kleine et al. 2012; Rothbaum et al. 2014), negative effects (Scheeringa and Weems 2014), or even deleterious effects (Litvin et al. 2007). These mixed results have suggested a number of potential issues that need consideration when designing treatment trials for DCS (and other putative extinction enhancing treatments): (1) are the effects of DCS more on *speed* of response rather than *magnitude* of response to exposure, two differing hypotheses that will require different experimental designs/analysis to probe efficacy; (2) what is the correct dosing/timing of treatment; (3) does DCS's cognitive enhancement promote inhibitory learning to the extinction context, which might subsequently contribute to contextual renewal of fear (Vervliet 2008); and (4) does DCS need to be targeted toward only the successful therapy sessions [for a detailed review, see Hofmann et al. (2015)]. This latter issue is because DCS is a broad cognitive enhancer, it can enhance both fear learning and extinction learning (Lee et al. 2006); thus, if the exposure session is unsuccessful in promoting extinction, it could instead promote reconsolidation (i.e., strengthening of conditioned fear to trauma memories and cues) that is then increased by DCS treatment. Thus far, however, predicting a "successful" session versus an unsuccessful one has been elusive. Alternatively, other groups are working to identify prescriptive variables that predict which subjects would most benefit from treatment, i.e., those with the most severe PTSD, specific symptom classes, or other traits (de Kleine et al. 2012, 2014).

It is worth noting that in humans, DCS has generally been found to be more efficacious in adjunct trials with exposure therapy in patient populations, compared to enhancing extinction of conditioned fear produced in the laboratory in healthy controls. One study (Kuriyama et al. 2011) out of 3 found DCS (and valproic acid) to enhance extinction. This study was the only one to utilize a reinstatement component, with DCS during extinction training affecting not within-session learning or recall, but instead suppressing reinstatement. DCS was ineffective in studies that limited their design to testing extinction acquisition and 24-h recall (Guastella et al. 2007; Klumpers et al. 2012). It has been suggested that this lack of translation of DCS effects on extinction in animals to extinction in healthy human subjects may be because extinction protocols in the laboratory are not probing "automatic" learned fear and extinction processes, but are instead governed by top-down executive functions (Grillon 2009). More recent studies, however, suggest that extinction in healthy controls is sensitive to putative extinction enhancing drugs such as cannabinoid receptor agonists and oxytocin (Acheson et al. 2013; Das et al. 2013; Eckstein et al. 2014; Rabinak et al. 2013), which suggests that these tests are "translational" in that they are sensitive to drugs that have shown efficacy in animal extinction studies (Singewald et al. 2015). Whether these drugs can then also make the leap to enhancement of exposure therapy or PTSD treatment is thus far mixed. Efficacy of cannabinoid receptor agonists for treating PTSD symptoms is promising (Cameron et al. 2014; Roitman et al. 2014), while oxytocin effects on exposure therapy are less clear (Acheson et al. 2013, 2015a; Guastella et al. 2009; Acheson and Risbrough 2015).

2.2.3 Is Fear Extinction Sensitive to Drugs that Are Effective for PTSD?

Although the bulk of pharmacology directed at extinction processes has been of drugs that are hypothesized to specifically act on this mechanism, it is fair to ask whether extinction is sensitive to current treatments. Chronic fluoxetine in rodents facilitates extinction learning and extinction memory recall, particularly in females (Deschaux et al. 2011; Fitzgerald et al. 2014; Lebron-Milad et al. 2013), and escitalopram enhances extinction in healthy humans (Bui et al. 2013), suggesting that examining effects of a drug on extinction may predict efficacy as an overall treatment beyond use as an adjunctive treatment with therapy. Paroxetine transiently enhanced effects of exposure therapy (Schneier et al. 2012); however, other studies show no efficacy of SSRIs to enhance exposure therapy in PTSD (Foa et al. 2005; Hetrick et al. 2010). It should be noted that when undergoing exposure therapy, many opportunities for exposure are outside of the therapist's office via "homework" developed to promote in vivo exposure in the patient's environment [in addition to imaginal exposure in prolonged exposure]; thus, a drug that can be given chronically may actually be more effective than a drug limited to exposure session treatments. Based on lessons learned from DCS in terms of potential unintentional enhancement of fear learning/reconsolidation, chronic treatment will depend on how selectively the drug acts on fear extinction mechanisms versus broader mechanisms of neural plasticity. (Besides its non-selective effects on extinction, DCS cannot be given chronically due to rapid tolerance.) An example of a potential target with more selective effects on extinction enhancement are agonists of the cannabinoid 1 receptor, in particular drugs that enhance endogenous ligand availability via inhibition of degradation (Steckler and Risbrough 2012).

2.2.4 Does fear extinction performance predict treatment response?

Currently, it is unknown whether extinction performance or other markers of extinction (e.g., ventral medial frontal cortex activation during recall) predict what type of treatment (e.g., pharmacology versus exposure therapy) or how much treatment (e.g., how many exposure sessions) might be most beneficial for patients. This question is of great interest in terms of supporting personalized medicine approaches and is actively being pursued by a number of research groups.

2.3 Reconsolidation and Reinstatement

Reconsolidation occurs when a memory is reactivated resulting in a period of transient lability of the underlying neuroplastic mechanisms supporting the

memory. During reconsolidation, old memories can be strengthened or disrupted by drugs that modulate consolidation mechanisms. The best characterized manipulation of reconsolidation of conditioned fear is via noradrenergic manipulations, with propranolol, a beta-adrenergic receptor antagonist, disrupting reconsolidation and subsequent conditioned fear responses in both animals and humans [for review see Otis et al. (2015)]. A recent meta-analysis indicates that propranolol is effective for blocking both consolidation and reconsolidation of fear memories in healthy humans (Lonergan et al. 2013). Recent studies however suggest that experimental design may be critical, with efficacy of propranolol given before memory reactivation having limited effect (Wood et al. 2015). Sevenster and colleagues showed that propranolol effects were only observable in conditions in which reconsolidation occurred under prediction uncertainty (i.e., the CS+ may or may not be followed by the US), suggesting that reconsolidation only occurs if the memory is actively being updated with new information (Sevenster et al. 2012). This group also cleverly showed that reconsolidation can be triggered not just by the specific CS+, but also by a semantically similar stimulus. Memory reactivation by semantically similar stimuli was sensitive to propranolol disruption (Soeter and Kindt 2015). This finding supports the feasibility of reconsolidation-based therapy, given the difficulty in accurately reconstructing trauma specific cues.

Reinstatement is when previously extinguished conditioned responding is "reinstated" after re-exposure to a US (Rescorla and Heth 1975). This phenomenon supports the now established view that extinction training does not "erase" the fear memory, but instead creates a competing CS–"No US" association with the original CS–US association. This CS–"No-US" association is further complicated by its dependence upon the extinction training context (Bouton 2014; Bouton and Todd 2014.) Studies of fear reinstatement in humans are relatively new and thus far primarily in healthy human controls (Dirikx et al. 2007; Hermans et al. 2005; Neumann 2008; Sokol and Lovibond 2012). Preliminary evidence suggests that cannabinoid receptor agonists given during or immediately after extinction training may suppress reinstatement (Das et al. 2013). There is an excellent review of current findings, methodology, and considerations for developing reinstatement protocols for drug development from the Lonsdorf laboratory (Haaker et al. 2014).

2.4 *Contextual Modification and Generalization of Learned Fear and Extinction*

Pavlovian fear conditioning occurs not only to discrete cues associated with a trauma, but also to the context in which a trauma occurs. The definition of what constitutes an associative context remains broad, but typically includes at least one of the following qualities: (1) unpredictable prediction of the US; (2) longer duration than a common discrete CS; and (3) complex, multimodal features. Contexts have been operationalized in numerous ways in laboratory tasks,

including the experimental setting itself, a virtual reality setting, pictures of rooms, and simple cues with an unpredictable US association (e.g., Alvarez et al. 2011; Armony and Dolan 2001; Bouton et al. 2006; Glenn et al. 2014; Grillon 2002; Effting and Kindt 2007; Neumann et al. 2007).

2.4.1 Do PTSD Patients Have Altered Contextual Fear Learning?

There is substantial research on contextual fear learning in animal models of PTSD (e.g., Daskalakis et al. 2013), though laboratory research on contextual learning in PTSD patients remains limited. Elevated startle response to unpredictable contextual threat has been found in PTSD patients (Grillon et al. 2009a, b). This finding suggests that PTSD patients may have elevated sensitivity to unpredictable threat, which contributes to sustained tonic "anxiety" responding, associated with activity in the bed nucleus of the stria terminalis (Walker et al. 2003).

Successful fear learning about multimodal contextual features depends upon configural processing in which a single configural representation binds together numerous co-occurring contextual elements (e.g., Rudy et al. 2004). Configural representation is a hippocampus-dependent learning process supporting identification of whether a context is similar ("pattern completion") or dissimilar ("pattern separation") to a previously encountered context. Impaired configural processing of a traumatic context has been theorized to contribute to contextual overgeneralization of fear experienced in PTSD (Acheson et al. 2012a, b; Glenn et al. 2014). Few, if any, studies have directly examined configural fear learning processes in PTSD patients. A fear conditioning study using two-dimensional images of similar-looking rooms as distinct contexts found that PTSD patients demonstrated poorer differentiation than healthy controls between threat versus safe contexts in contingency ratings (Steiger et al. 2015). The authors note that the contextual stimuli used in this study were relatively simple static photographs of rooms (hallway, library) so contextual differentiation in this task may not have required configural processing. For example, it would have been possible to distinguish between contexts by attending to a single contextual element (the presence or absence of books on the walls) without considering the overall configurations, meaning that this task did not necessarily evaluate hippocampus-dependent contextual fear learning deficits in PTSD. Configural learning deficits have been found in PTSD combat veterans, and their non-trauma exposed twins relative to non-PTSD combat veterans (Gilbertson et al. 2007), though this study utilized a "cube and paper test" which did not examine contextual learning in relation to fear conditioning.

PTSD patients have been shown to exhibit deficient extinction of contextual fear (Steiger et al. 2015). There is an extensive literature on contextual modulation of extinction and return of fear in patients with anxiety disorders (e.g., Vervliet et al. 2013) and some evidence of altered contextual modulation of extinction in PTSD patients (Rougemont-Bücking et al. 2011).

2.4.2 Do PTSD Patients Have Altered Generalization of Fear?

Generalization of fear is the process whereby conditioned fear responding occurs not only to stimuli directly associated with the US, but also to stimuli similar to the CS (e.g., Dunsmoor and Paz 2015; Dymond et al. 2014). Fear generalization is a particularly relevant process for PTSD as much of the fear experienced by PTSD patients is triggered by encountering generalization stimuli (GS) which act as reminders of the trauma due to similarity to the original conditional stimuli, rather than through encountering the actual stimuli directly involved in the trauma. Laboratory assessment of fear generalization typically includes two phases: (1) a standard differential fear conditioning phase involving both a CS+ repeatedly predictive of an aversive US and a CS− never paired with the US and (2) a generalization test measuring responding to GSs with varying levels of similarity or relatedness to the CS+. The CS+ and CS− in generalization tasks commonly differ along a particular observable gradient, such as size or color (e.g., small circle/large circle, black square/white square), but there has been extensive research on non-perceptual forms of generalization as well including category-based, semantic, and symbolic fear generalization [for reviews see Dunsmoor and Paz (2015), and Dymond et al. (2014)]. Through such methodology, a generalization gradient is generated, indicating the extent to which strong conditional responding occurs only to GSs very similar to the CS+ (steep gradient) versus responding to GSs with high and low CS+ similarity (shallow gradient).

Despite a robust literature on fear generalization and a sound theoretical basis for the relevance of generalization to PTSD, laboratory research on fear generalization in PTSD patients is extremely limited. Relative to healthy controls, PTSD patients as well as panic disorder and generalized anxiety disorder patients show shallow fear generalization gradients, indicating overgeneralization of conditioned fear (Lissek et al. 2010, 2014a; Lissek and van Meurs 2014). These data are in line with findings that subjects with PTSD do not show physiological discrimination between CS+ and CS− cues, even though they report contingency awareness perfectly accurately (Acheson et al. 2015b; Jovanovic et al. 2012). This deficit in "automatic" fear discrimination between safe and threat cues appears to be specific to PTSD symptoms compared to generalized anxiety or depression symptoms (Acheson et al. 2015b). Thus, pharmacological enhancement of cue discrimination may be an effective strategy for a number of anxiety disorders, not just PTSD.

Recent neural models of fear generalization identify hippocampal substrates involved in both pattern completion (CA3 region, involved in recognizing a GS as similar to previously encountered CS+) and pattern separation (i.e., dentate gyrus, involved in recognizing a GS as dissimilar from previously encountered CS+), while subregions of the central and lateral amygdala, the bed nucleus of the stria terminalis, and the ventromedial prefrontral cortex have been implicated in expression of generalized fear (Besnard and Sahay 2015; Dunsmoor and Paz 2015; Lissek et al. 2014b). It is noteworthy that models of pattern completion and separation in fear generalization are similar to hippocampus-centered models of contextual fear learning (Kheirbek et al. 2012; Rudy et al. 2004). Configural learning is

thought to encode complex, multimodal features of the trauma environment, however, while the term fear generalization is typically used in relation to discrimination across relatively simple stimulus gradients. Greater generalization of simple stimuli may be expected when configural learning of contextual information is impaired such that context learning must be learned through elemental representation, a learning process in which individual contextual elements are not bound together but independently associated with the negative outcome (Maren et al. 1997; Rudy et al. 2004).

2.4.3 Are Contextual Fear Learning and Fear Generalization Processes Sensitive to Drugs that Are Effective for PTSD?

No research to date has examined drug effects on contextual fear learning or fear generalization processes in PTSD patients, though preliminary experimental research suggests that acute glucose consumption may enhance retention of differential configural fear learning (Glenn et al. 2014). In healthy subjects, acute administration of 1 mg of the benzodiazepine alprazolam reduced sustained startle responding in both predictable and unpredictable "context" periods, but did not alter responding to discrete cues associated with predictable and unpredictable threat (Grillon et al. 2006). These findings tentatively suggest that acute benzodiazepine administration might reduce sustained contextual anxiety in PTSD patients, though they do not indicate treatment effects for sensitivity to unpredictable threat.

Findings from animal research are mixed regarding medication effects on contextual fear learning. One recent review concludes that both acute and chronic SSRI administration reduce plasticity in the hippocampus and decrease expression of contextual fear learning (Burghardt and Bauer 2013), while another review suggests that chronic antidepressant administration enhances configural learning processes through promotion of neurogenesis in the dentate gyrus (Castren and Hen 2013). Given the involvement of pattern separation and pattern completion in both fear generalization and contextual fear learning, there is reason to expect that drugs promoting neurogenesis in the dentate gyrus might be used to both improve configural learning of contextual information and decrease overgeneralization of feared stimuli in PTSD patients (Besnard and Sahay 2015; Castren and Hen 2013). No research has directly examined drug modulation of contextual fear extinction in PTSD, though it has been argued that DCS promotes contextual safety learning (Vervliet 2008; Woods and Bouton 2006). Theoretically, drugs that improve pattern completion and separation could be used prophylactically during or immediately following trauma to improve specificity of learning and prevent overgeneralization of contextual or discrete fear (Glenn et al. 2014). Conversely, such drugs may be contraindicated for use in conjunction with exposure therapy for PTSD and other anxiety disorders given concerns that greater contextual specificity of fear extinction learning increases the probability of contextually mediated renewal of fear (Bouton et al. 2006; Vervliet et al. 2013).

2.5 *Practical Considerations When Using Learned Fear Processes as a Marker of Drug Efficacy*

Because fear conditioning involves active learning, consolidation, and recall, treatment regimens will have critical consequences on how drug effects can be interpreted. Whether a treatment is hypothesized to block fear consolidation (i.e., potential utility as prophylactic) versus simply block fear expression (i.e., therapeutic utility) is a key component to appropriate study design. Sub-chronic or chronic dosing regimens are the norm for initial early phase studies. Animal studies of when the drug is most effective, either at blocking fear conditioning or at expression, are critical in planning interpretable fear conditioning studies across the dosing timeline (e.g., condition before or during dosing to test drug effects on expression versus conditioning, respectively). There is a similar issue for studies of extinction, with a note of caution from our own studies on oxytocin effects on extinction. To test the effects of oxytocin on extinction, we employed a common 2-day protocol; on the first day, fear conditioning was followed by drug treatment and subsequent extinction training trials, with the fear recall test 24 h later. We found a significant increase in extinction recall in the oxytocin group (i.e., less fear than placebo), suggesting a potential enhancement of extinction encoding/consolidation (Acheson et al. 2013). A recent study using fMRI with a very similar 1-day design of fear conditioning being followed by treatment and extinction training confirmed that within-session extinction could be enhanced by pretraining oxytocin (Eckstein et al. 2014). These findings supported subsequent examination of oxytocin to enhance extinction-based therapy. However, a preliminary study we conducted in spider phobia subjects indicated that oxytocin treatment has the opposite effect than expected, and it interfered with exposure therapy effects, with placebo treated subjects exhibiting better long-term reductions in phobia symptoms than the oxytocin-treated subjects (Acheson et al. 2015a). It is not clear whether this lack of translation is due to a potential design problem in the exposure therapy trial, including too short an exposure regimen (1 session), or whether our interpretation of oxytocin effects in laboratory-based tasks was erroneous. An alternate interpretation is that oxytocin treatment, administered soon after fear conditioning, could instead have disrupted consolidation of the fear memory (Acheson and Risbrough 2015). Thus, what was interpreted as effects on improving extinction training/recall may have actually been interfered with fear consolidation, and only a test design in which conditioning and extinction are separated more widely in time (i.e., 24 h) can be sure of the correct interpretation. A 3-day design, with conditioning, extinction, and recall on separate days, is of course more difficult in terms of retraining subjects; however, such a design will greatly enhance accurate interpretation.

An additional concern in terms of drugs effects on fear extinction is whether inhibitory learning processes are expedited (i.e., faster reduction in fear) or made more robust to relapse. It has recently been noted that in exposure therapy, the extent to which reductions in fear are long-lasting and resistant to relapse may be of greater clinical value than the sheer magnitude of decrease in fear (Vervliet et al. 2013).

This same consideration should be given to evaluating drugs targeting fear extinction, with designs that incorporate assessment of long-term recall and resistance to return of fear.

3 Summary

In conclusion, the use of laboratory-based measures of fear processes has offered the promise of exciting new targets for PTSD. Although the field continues to have gaps between findings in laboratory-based fear and effects in exposure-based therapy (e.g., DCS and oxytocin), parallel work in better defining DCS effects on fear processes and how these effects might both impede and facilitate exposure are currently underway. Using laboratory measures of fear learning processes to predict treatment response in patients is also potential evolution of the utility of fear-based tasks in informing treatment approaches. As discussed above, careful evaluation of study design and treatment approaches within the fear learning/extinction continuum will be critical in early-phase proof-of-concept studies. Designing studies with assessment of long-term recall/resistance to reinstatement will also be critical in evaluating drug effects either on fear consolidation (inhibitory) or on fear extinction (enhancement or improved generalization) for the chances of efficacy in the clinic.

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ARMY STARRS
Army Study to Assess Risk and Resilience in Servicemembers

Army STARRS Collaboration with Marine Corps

Builds on Marine Resiliency Study

Funded by the Marine Corps

Research team from UCSD & San Diego VA

Many elements similar to Army STARRS (Surveys, Biomarkers, Neurocognitive Measures)

Additional measures (Fear Conditioning, Cardiovascular Assessment, Clinical Evaluation)

Projects

Pre/Post-deployment Studies

Assessing both Infantry & Combat Engineers – ongoing

Demonstration Projects to inform Army STARRS

- Modify Army STARRS neurocognitive tests for use in Marines – done
- Genetic/epigenetic markers of risk/resilience post-deployment – ongoing
- Metabolomic & oxidative stress markers of risk & resilience – approved
- PTSD/TBI neuroimaging study in EOD personnel – under review at NIMH

Characterization of Cerebrospinal Fluid (CSF) and Plasma NPY Levels in Normal Volunteers over a 24-h Timeframe

Dewleen G Baker[1,2,3], Richard L Hauger[1,2,3], Tobias Moeller Bertram[1,2,3], Piyush M Patel[2,3], Donald A Barkauskas[4], Paul Clopton[3], Thomas D Geracioti Jr[5], Daniel T O'Connor[2], Caroline M Nievergelt[2,3]

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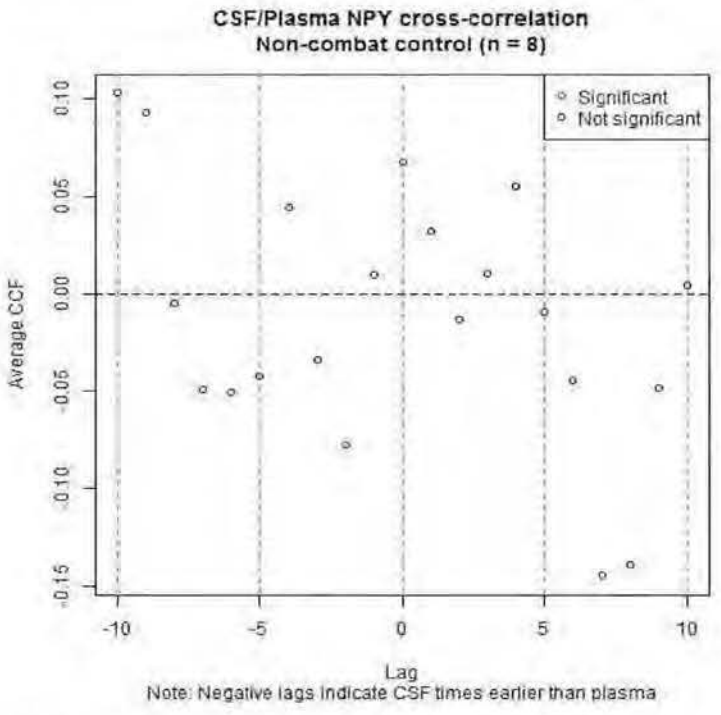
BACKGROUND: Neuropeptide (NPY) is abundant in mammals, where it contributes to diverse functions centrally and peripherally. Its wide brain distribution provides a plausible substrate for its relevance to stress. Despite an increasing interest in NPY as a moderator of stress in humans, the extent to which plasma and cerebrospinal fluid NPY concentrations are accurate reflections of each other is poorly understood.

OBJECTIVES: The objective of this study is to more thoroughly characterize NPY CSF/plasma concentration relationships.

METHODS AND RESULTS: Eleven healthy male civilian study volunteers participated in a 24-h serial CSF and plasma sampling study. All met study inclusion criteria based on physical examination, mental health (DSM-IV) interviews. At 8AM the morning after admission, a catheter was inserted via a 17-gauge Touhy needle into the L4-L5 lumbar space. Between 11AM on day one CSF (and plasma, from an indwelling venous catheter) were collected. All fluids were stored at -80^{O}C until assay of (hourly) samples. As observed in prior studies, group mean (SE) CSF NPY (cNPY) levels [792.1 (7.80) pg/mL] were higher than plasma (pNPY) levels [220.0 (3.63) pg/mL]. Lagged cross-correlation (CFF) analysis showed no statistically significant cross-correlations between cNPY and pNPY at the $p \geq .05$ level [see Figure]. Average pNPY/cNPY concentration ratios ranged from .20 to .40 across study subjects. The pNPY/cNPY ratios appear to be individual specific and consistent across the 24-h time period. cNPY circadian components were not



detectable owing to a large positive linear trend.

CONCLUSIONS: These findings suggest that interpretation of the physiological significance of plasma NPY concentrations in human NPY stress or resilience studies must account for the lack of correlation between plasma and CSF NPY concentrations.

NPY: Low Cerebrospinal Fluid (CSF) Levels in Posttraumatic Stress Disorder in Comparison to Combat and Civilian, non-combat Control Subjects

Dewleen G Baker[1,2,3], Richard L Hauger[1,2,3], Tobias Moeller-Bertram[1,2,3], Piyush M Patel[2,3], Donald A Barkauskas[4], Paul Clopton[3], Thomas D Geracioti[5], Daniel T O'Connor[2], Caroline M Nievergelt[2,3]

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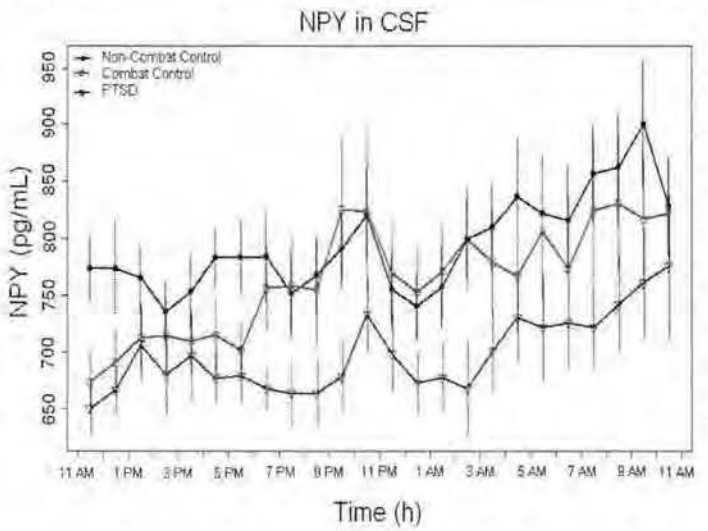
BACKGROUND: The NPY system is associated with behavioral resilience to stress exposure in an animal model of Posttraumatic Stress Disorder (PTSD); its role in the humans with PTSD is being explored.

OBJECTIVES: The key objective of this 24-hour serial CSF study of NPY in PTSD was to replicate and expand upon a prior single time point PTSD study showing low CSF NPY, by evaluating basal 24-h NPY concentrations across three study groups, civilian volunteers and combatants of the Iraq and Afghanistan conflicts with and without PTSD.

METHODS AND RESULTS: Participants were 26 age-matched, males, 12 with PTSD, 14 healthy deployed and 11 civilians. After CSF catheter insertion, beginning at 11AM on study day one, CSF was collected every half hour for 24-h from an indwelling CSF catheter, as was plasma from a venous catheter. Fluids were stored at -80^{O}C until assay. Group demographic comparisons using FDR-adjusted *p*-values showed no statistically significant differences across study groups regard age or BMI. Using linear mixed-effect models, differences in NPY-CSF concentrations were statistically significant [*p*-value for existence of group effect 0.0234; the difference in levels between healthy civilian volunteers and PTSD subjects was statistically significant ($p = 0.012$) but deployed healthy subjects were not statistically significantly different from either of the other two groups]. Additionally, cNPY increased at an estimated rate of 5.5 pg/mL/hour ($p < 0.0001$) but tests for interaction showed no statistically significant differences in the linear trends among the three subject groups.



CONCLUSIONS: These findings suggest that NPY may be involved in behavioral resilience to stress in humans, thus may be a good target for interventions for prevention or early intervention.

Prospective assessment of psychophysiological risk factors for PTSD

Risbrough V; Baker D, Nievergelt C; Litz B; Nash W; Perez J; Geyer M

University of California San Diego; National Center for PTSD, Boston VA

Rationale/Statement of the Problem: There is an urgent need to develop biological and behavioral predictors of PTSD risk/resilience in individuals with high trauma exposure such as active duty military. First we will briefly review psychophysiological risk factors for PTSD. Second we will describe preliminary data from a prospective study of active duty Marines examining psychophysiological responses before and after deployment to Iraq or Afghanistan. Third we will discuss our cross-species work in animal models of PTSD risk/resilience to inform these study findings.

Methods: This study was conducted as part of a 4 hr battery (clinical, psychosocial, laboratory and psychophysiological assessments) conducted both before, and 3 mo and 6 mo after deployment (Marine Resiliency Study) in >2500 Marines. Here we examined (1) effect of deployment overall on physiological reactivity measures on baseline startle, prepulse inhibition and affective modulation of startle), and (2), comparison of pre-deployment startle reactivity across subjects matched for combat exposure with and without PTSD symptoms 3 mo post-deployment.

Results: We observed small but significant increases in baseline startle and increases in prepulse inhibition after deployment. Startle potentiation to aversive images was also significantly increased after deployment. Importantly, baseline startle magnitude *before* deployment was significantly greater in subjects that went on to develop PTSD symptoms after deployment compared to their combat-matched controls.

Conclusions: These results support previous reports suggesting that startle reactivity may probe trait biological processes that confer risk for PTSD symptoms. To complement these findings we (1) are conducting a similar prospective study to determine if fear conditioning and extinction performance predicts deployment-related stress disorders and (2) have developed a homologous rodent model to aid identification of potential epigenetic mechanisms underlying psychophysiological and fear processing risk factors.

Magnetoencephalography (MEG) source imaging markers for mTBI and PTSD

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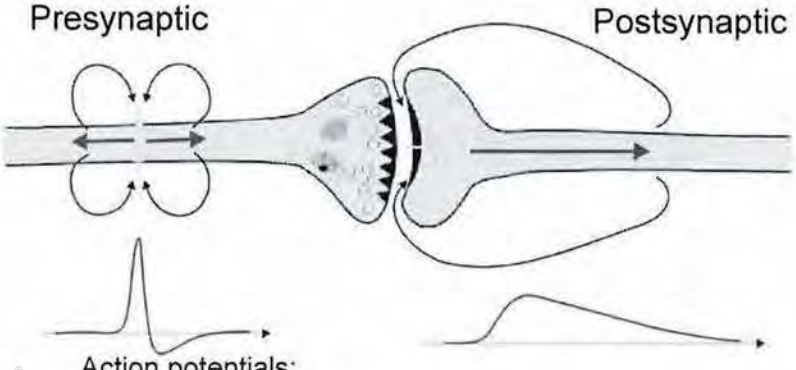
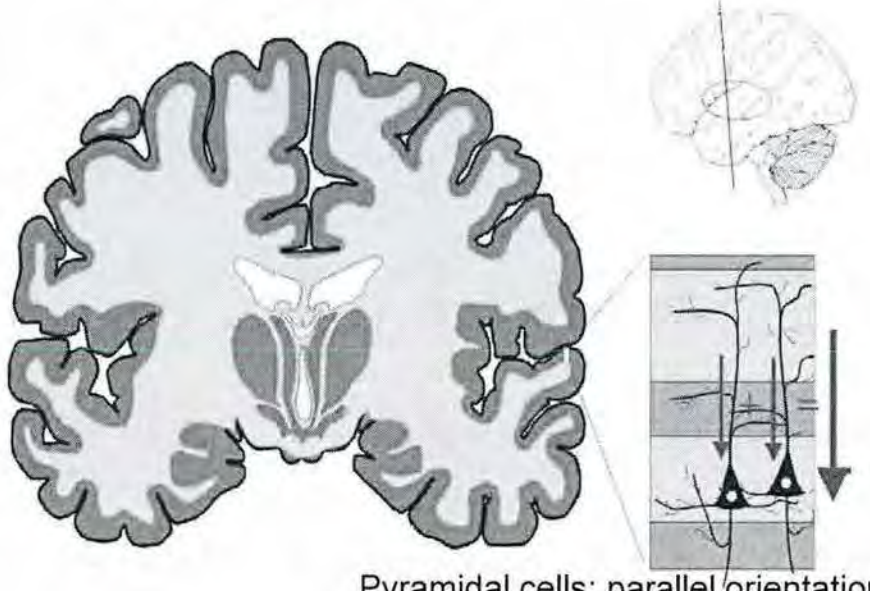
The generation of MEG signal from neuronal current in gray-matter neurons in cortex

Neuronal currents in axons and dendrites

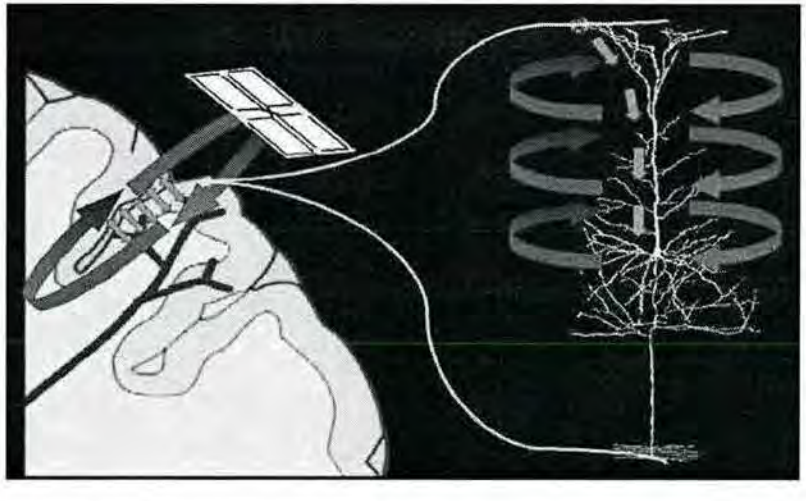


- Action potentials:
  - Fast: no/little temporal summation
  - Cancellation: fields diminish rapidly

Parallel dendrites

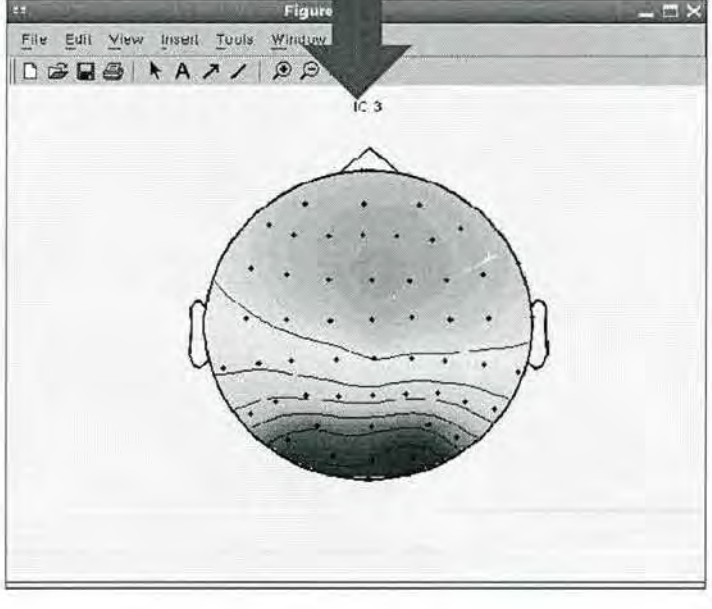


Pyramidal cells: parallel orientation
=> spatial summation



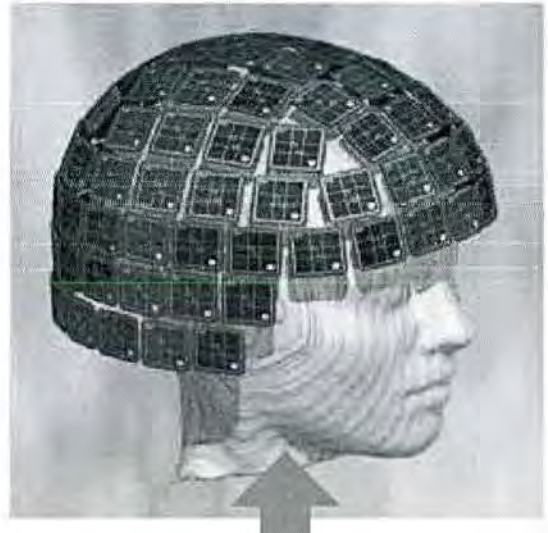
EEG vs. MEG Technology

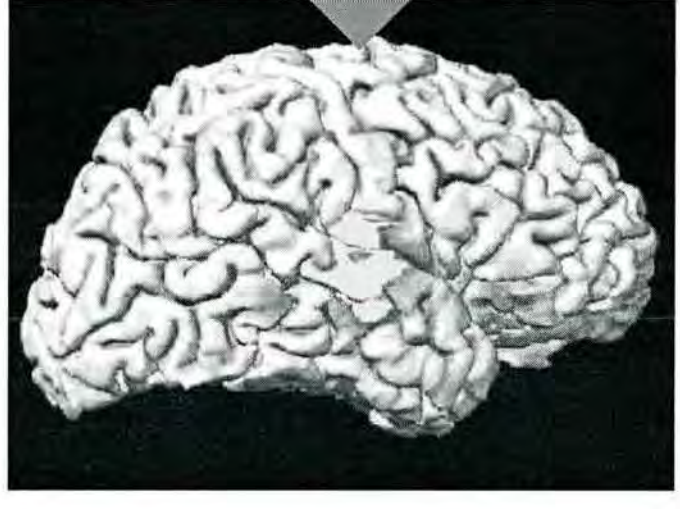
EEG



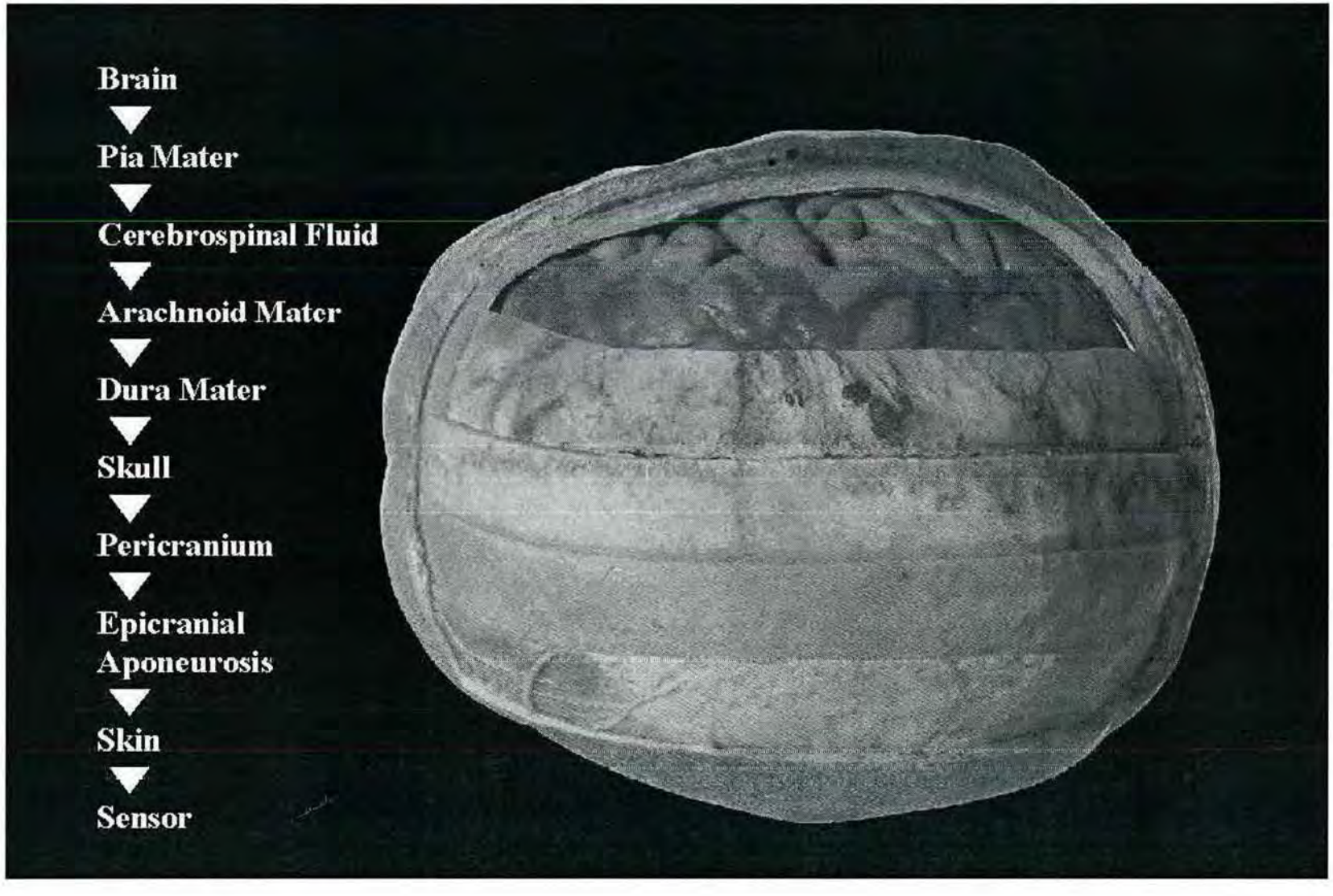
MEG

MEG SQUID Sensor Array



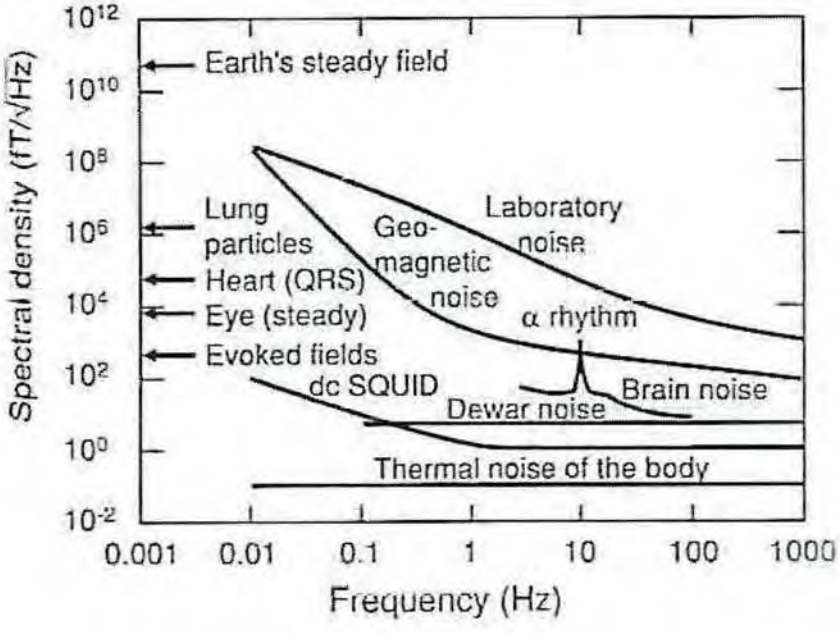


MEG's better spatial localization accuracy (2-3 mm) than high density EEG (in cm) is due to MEG's insensitivity to conductivity profile of the head tissues



Non-invasive MEG Technique with 1 ms Temporal Resolution and several mm Spatial Resolution in Cortex

MEG signal is weak



IMEDCO Multiple Layer Magnetic Shielded Room Installed at UCSD

Shielding factors:

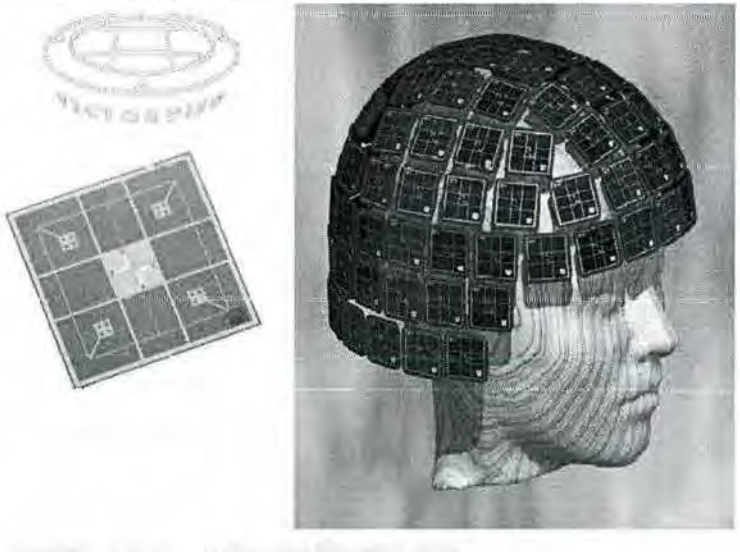
0.01Hz: 65dB

0.1 Hz: 73dB

1 Hz: 108dB

10Hz: 160dB

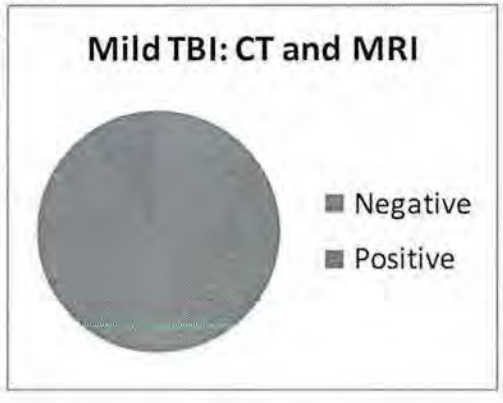
MEG SQUID Sensor Array



Elekta/Neuromag VectorView Whole Head MEG System with 306 Channels at the UCSD MEG Center

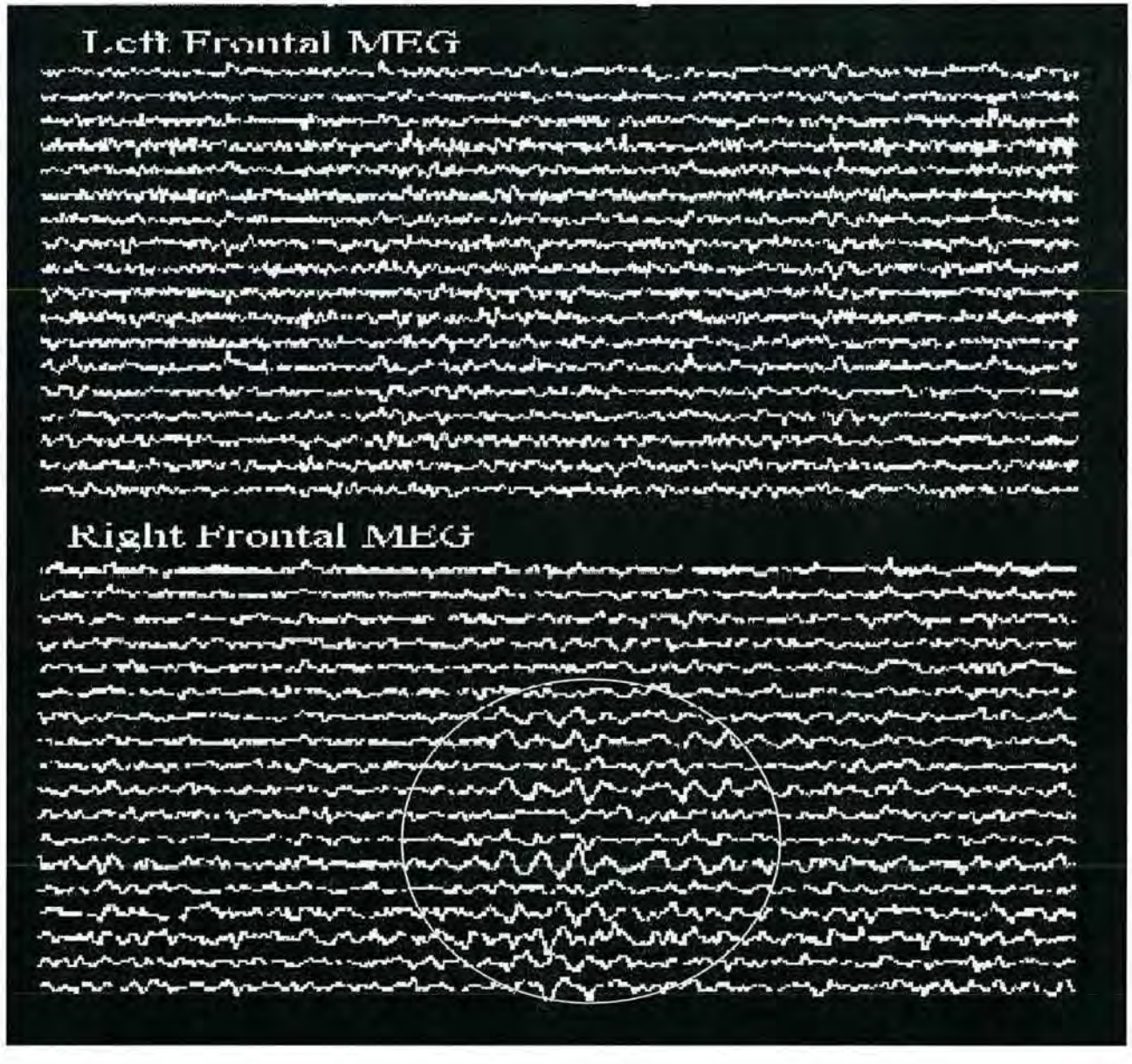
Mild TBI is often referred as *invisible* injuries: Detecting Mild TBI is Challenging using Conventional Neuroimaging Methods

- Traumatic brain injury (TBI) is a leading cause of sustained impairment in veterans, military personnel, and civilian populations.
- Mild TBI (mTBI): injuries are difficult to detect (injuries visible on only 10% of conventional MRIs or CTs).
- Axonal injury is a leading factor in mTBI. Conventional CT and MRI are mainly sensitive to blood product, and less sensitive to axonal damage itself, hence they underestimate the presence of axonal injury, especially in mild TBI cases.
- Injured brain tissues in mTBI patients generate pathological slow-wave magnetic signal that can be measured and localized by MEG (Lewine et al., 1999, 2007).
- Integrate gray-matter MEG slow-wave with white-matter diffusion tensor imaging (DTI) findings in mTBI (Huang et al., 2009)

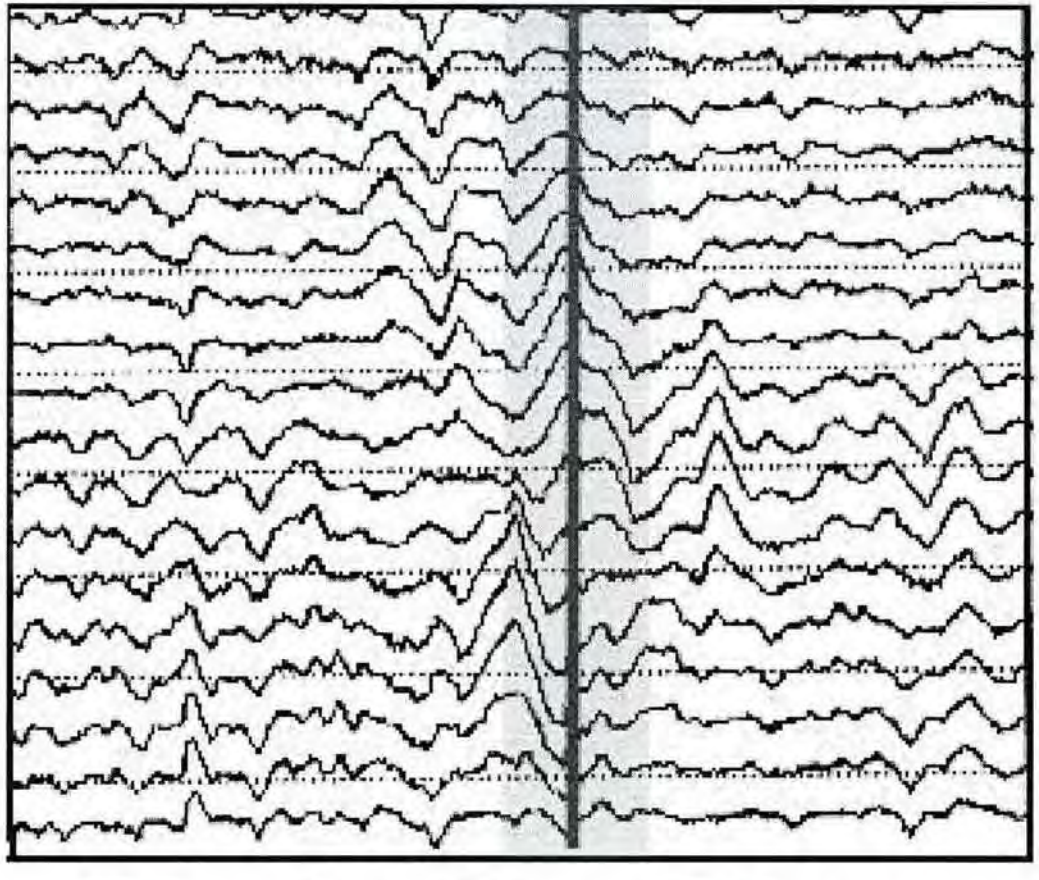


Abnormal Resting-state MEG Slow-waves in gray-matter (1-4 Hz, delta-waves) are Characteristics of Neurological Injuries in the Brain, resulting from axonal injury

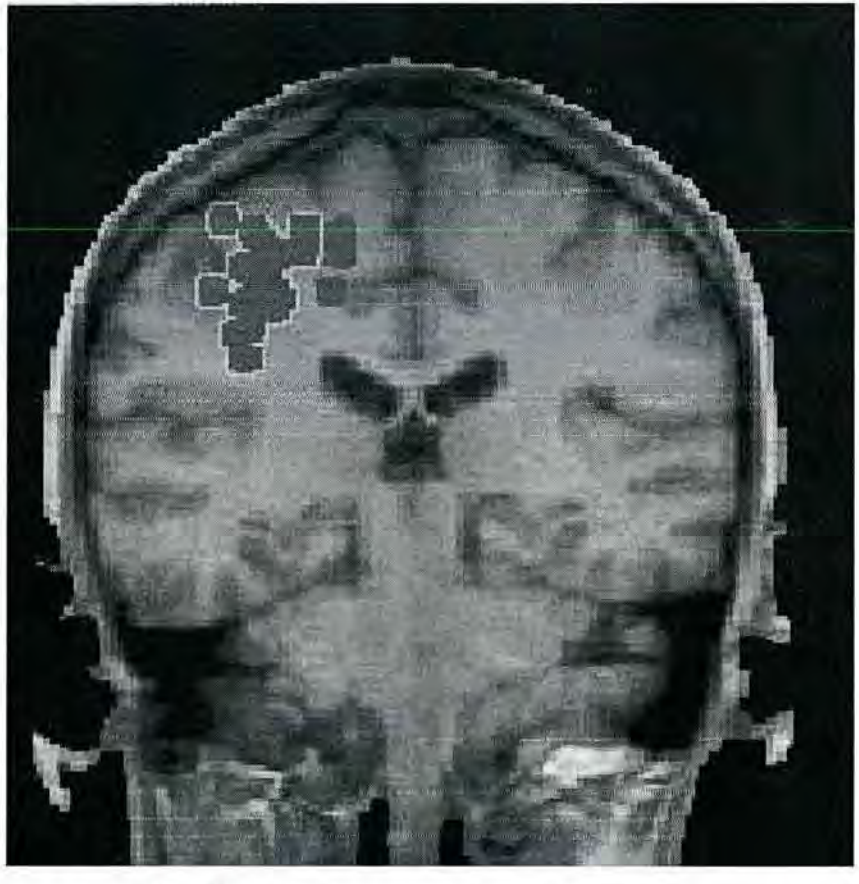
- Stroke
- Brain tumor
- Epilepsy
- Traumatic brain injury



Resting-state Slowing in mild head trauma patients with normal MRI



Dipolar Slow Wave



Mild Head Trauma

What is the neurophysiology for resting-state MEG slow-wave generation (1-4 Hz) in TBI?

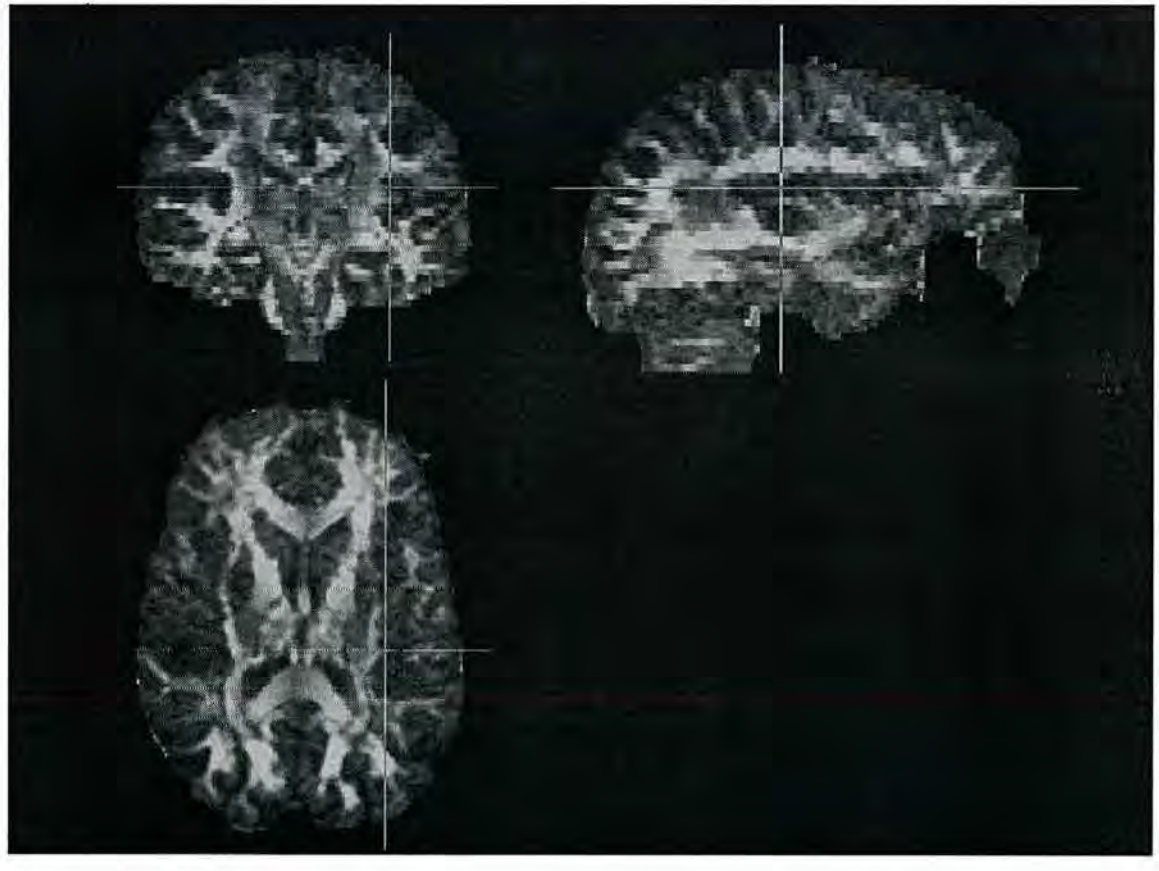
- Animal studies in cats revealed the slow-wave (delta-band 1-4Hz) were due to De-afferentation in gray-matter, caused by axonal lesions in white matter (Gloor et al., Neurology, 1977; Ball et al., Electroencephalogr. Clin. Neurophysiol., 1977).
- Is it possible that abnormal MEG slow-waves in mTBI patients are also due to de-afferentation from axonal injury?
- Our MEG-DTI integration study examines slow-wave in gray-matter and axonal injury in white matter (Huang et al., Journal of Neurotrauma, 2009)

Diffusion Tensor Imaging (DTI)

- DTI is an MR imaging technique based on the Brownian motion of water through tissues
- It measures how easy that water molecules move along the direction of white matter fibers versus the directions perpendicular to the fibers.
- TBI causes tissue shearing in the white matter fibers that leads to reduction of DTI signal.

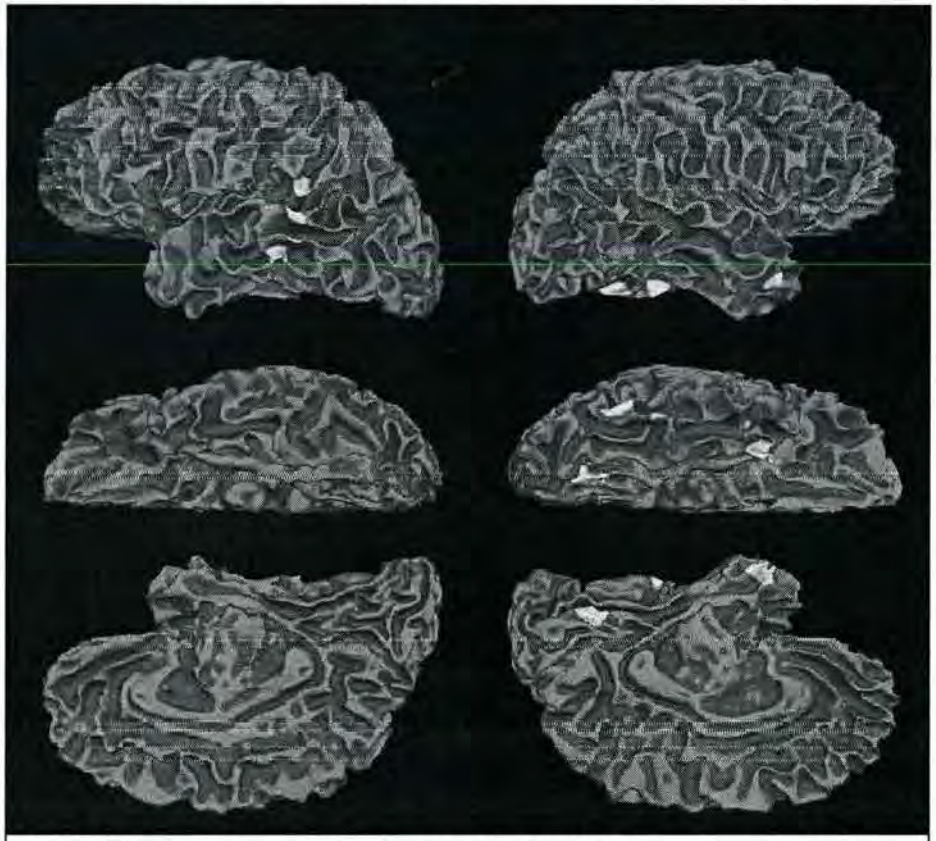
$$D = \begin{pmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{pmatrix}$$

$$FA = \frac{\sqrt{3\left[(\lambda_1 - \bar{\lambda})^2 + (\lambda_2 - \bar{\lambda})^2 + (\lambda_3 - \bar{\lambda})^2\right]}}{\sqrt{2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}}$$

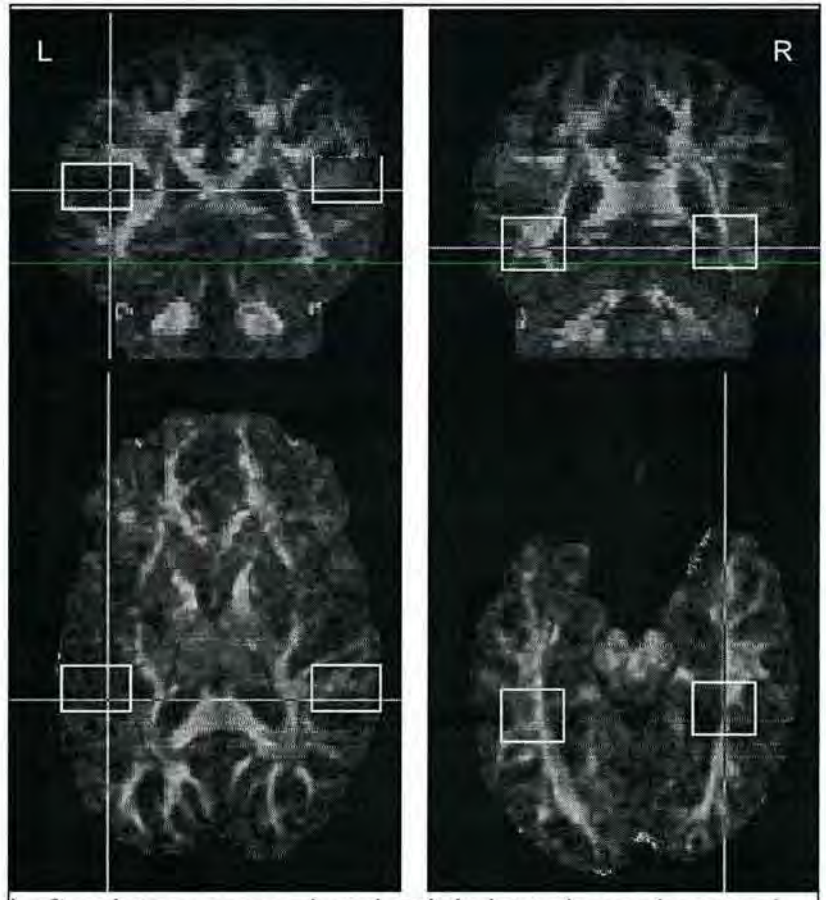


MEG-DTI Findings in Mild TBI due to Sport-related Accidents

History: 17-year old football player, 3 mTBIs. Symptoms: progressive headaches. dizziness, extreme fatigue while performing any mental task, altered sleep, memory problems, changes in speech.
Evaluation: Multiple CT & MRI scans negative.



rs-MEG results show abnormal slow-waves generated from two regions in a TBI patient: 1) left column -- left lateral superior-posterior temporal region, 2) right column --- right inferior-temporal areas. Color threshold $p<0.01$.



Left column: coronal and axial view show abnormal DTI in superior-posterior temporal lobe of the left hemisphere in a TBI patient. Right column: abnormal DTI in inferior-temporal lobe as part of the inferior longitudinal fasciculus of the right hemisphere.

Huang et al., J Neurotrauma. 2009 Aug;26(8):1213-1226.

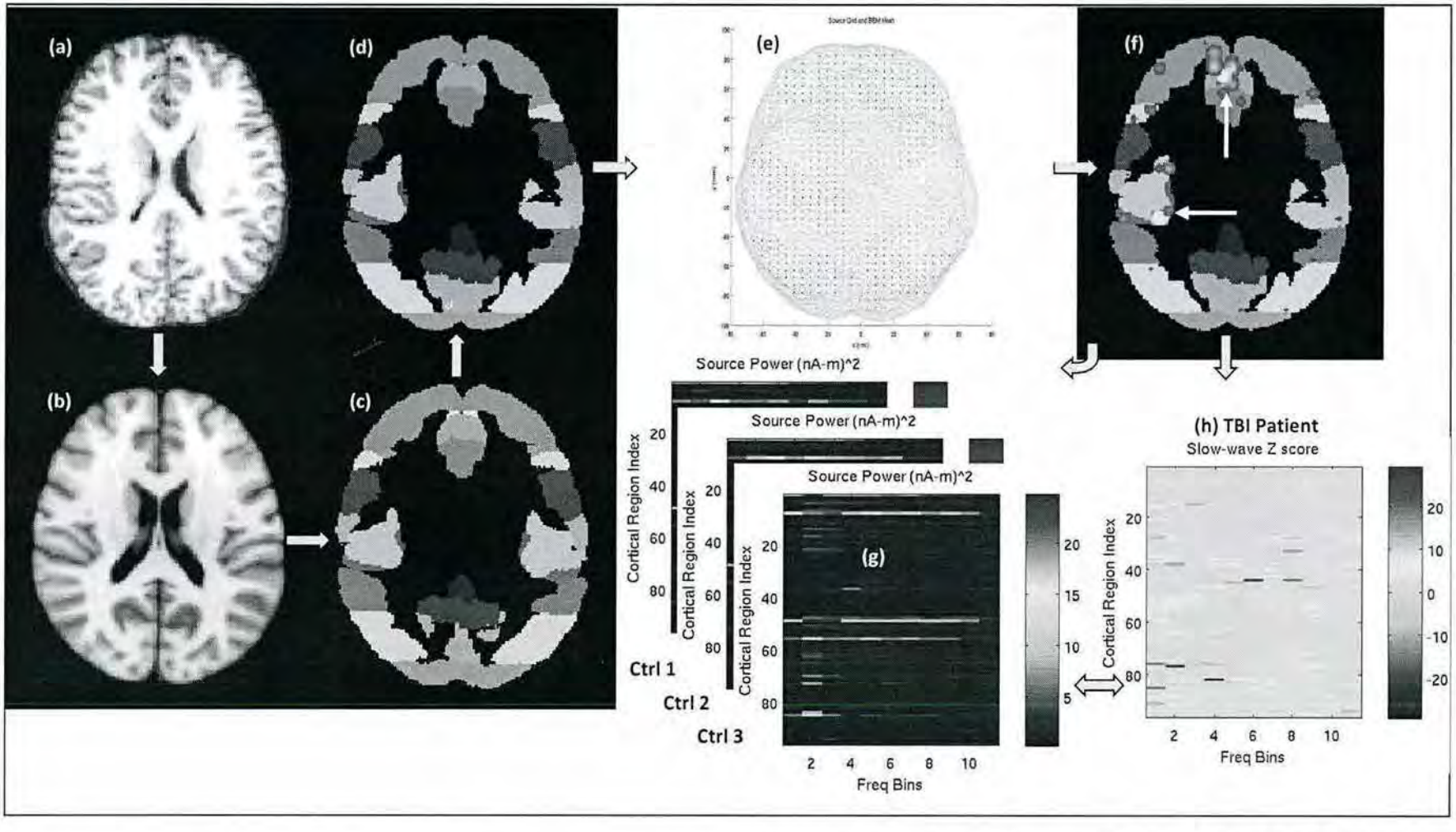
Examining the Positive Detection Rate of Mild TBI using resting-state MEG

- Resting-state MEG data (spontaneous recording with eyes-closed for slow-wave detection) were collected using the Elekta-Neuromag VectorView whole-head MEG system.
- Group 1 contains 23 mild TBI patients whose injuries were caused by blast, all with PCS;
- Group 2 contains the 22 mild TBI were injured with non-blast causes (i.e., motor vehicle accident, sports, and fall), all with PCS
- Group 3 contains 10 moderate TBI that were not blast-related, all with PCS.
- Group 4 contains 44 age-matched healthy control subjects.

Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG Slow-wave Source Imaging

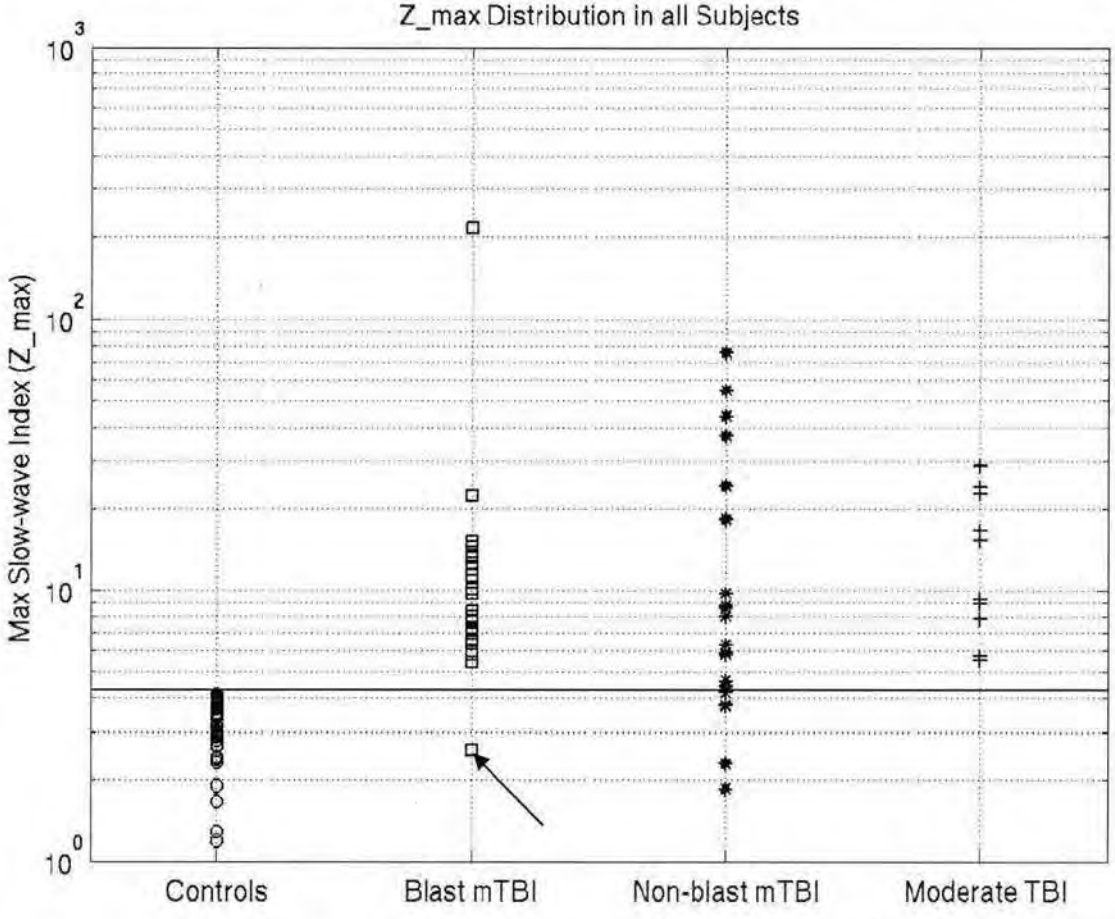
- Resting-state MEG data were analyzed using our new improved frequency-domain VESTAL method to obtain the source images for the low-frequency range (1-4Hz). Normative Database from healthy control subjects were used to detect abnormal slow-wave generation in TBI patients.



Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG Slow-wave Positive Detection Rates for TBI

- MEG positive-finding rates for different TBI groups were calculated at the threshold of 0% false-positive rate in healthy control subjects.
  - In the mild TBI group caused by blast, the MEG positive-finding rates was **96%**.
  - In the mild TBI group with non-blast causes, the MEG positive-finding rates was **77%**.
  - In the moderate TBI group, the MEG positive-finding rate was **100%**.
  - In the combined mild TBI group (blast+non-blast), the MEG positive-finding rates was **87%**.



Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG Slow-wave Exam Correlates with Post-concussive Symptoms

- $N_{slow\text{-}wave\_sum}$ is significantly correlated with N_{PCS_sum} ($r=+0.27$, $p<0.05$) in 55 TBI patients
- Regarding Individual PCS, $N_{slow\text{-}wave\_sum}$ significantly correlated with Personality Changes (e.g., social problems) ($r=+0.32$, $p<0.05$), Apathy, ($r=+0.36$, $p<0.01$), and other visual difficulties ($r=+0.27$, $p<0.05$).

Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG slow-wave imaging marker for TBI

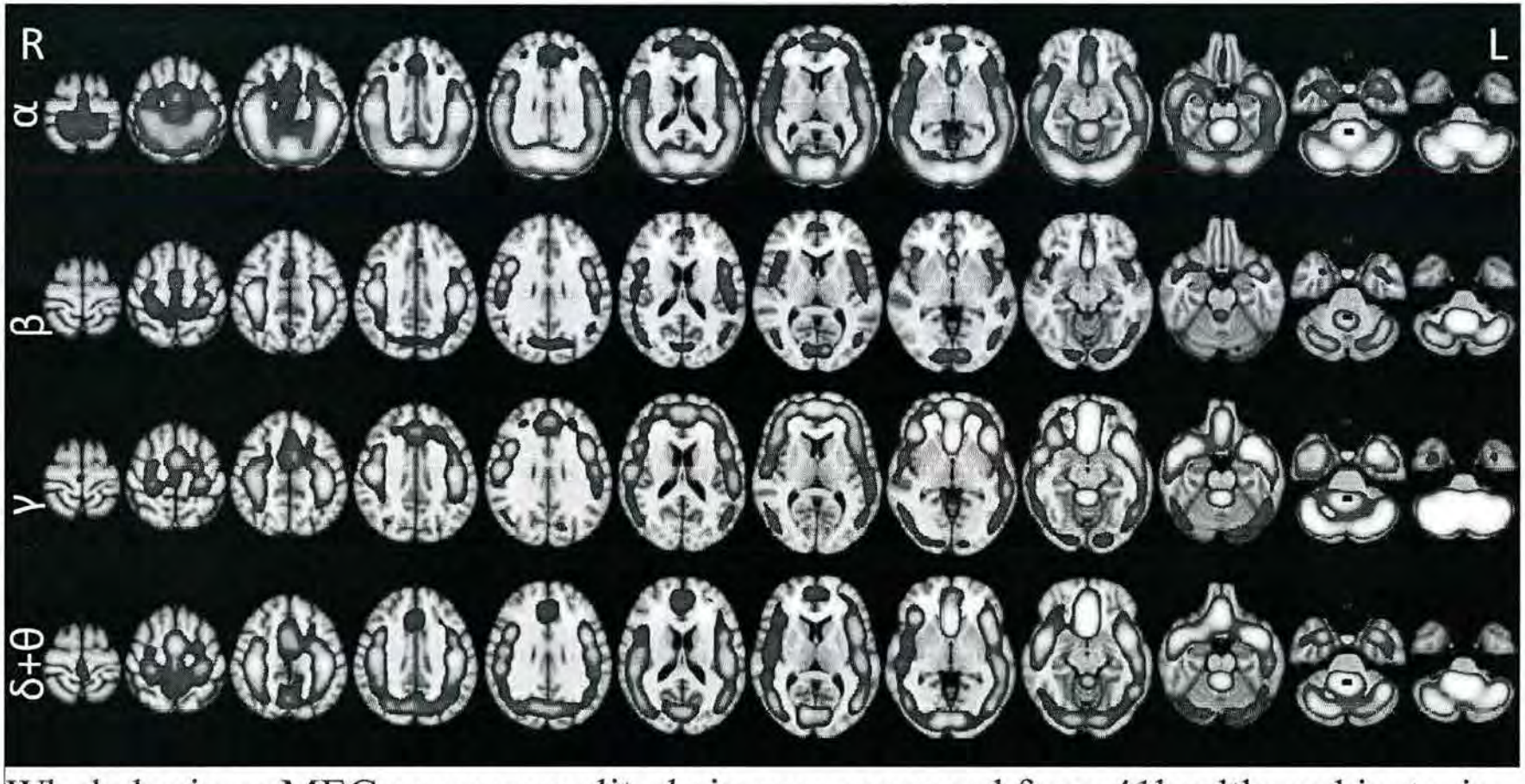
- Positive-detection Rates: MEG slow-waves in TBI patients has **87**% positive-finding rate in mild TBI and **100**% for moderate TBI groups.
- MEG slow-wave findings correlate with post-concussive symptoms

Huang et al., NeuroImage, 2012, 61(4):1067-1082.

Developing MEG source imaging marker for PTSD: Study I

- Resting-state MEG recording with eyes-closed
- 44 Healthy Controls
- 21 Veterans diagnosed with PTSD (CAPS total: 53-92)
- MEG source imaging for gamma band activity (30-100 Hz)

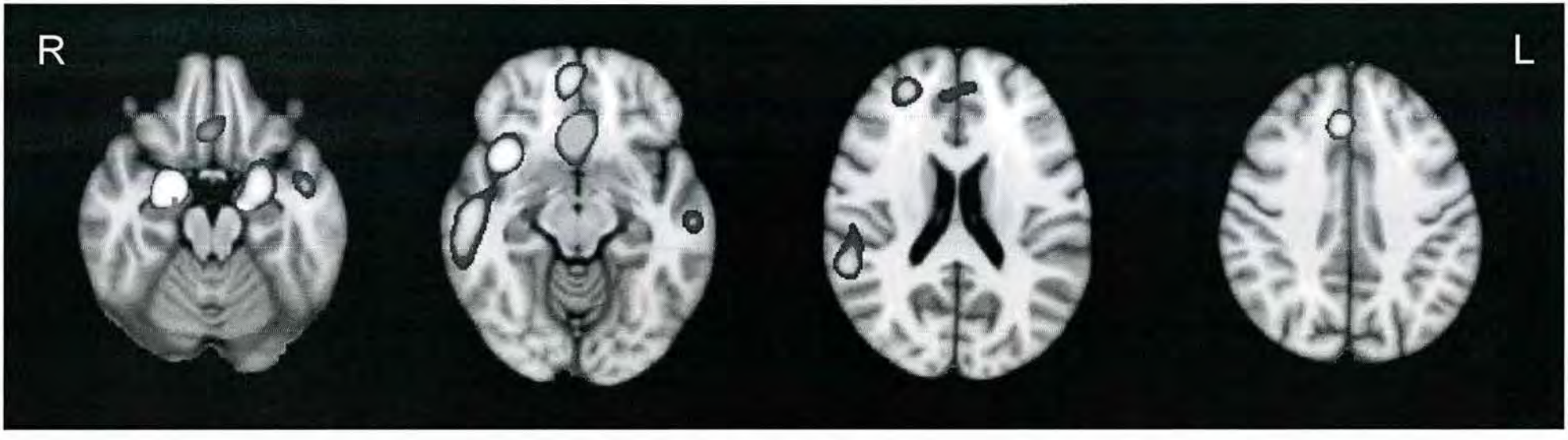
First Whole-head Source Amplitude Images of Brain Rhythms for Different Frequency Bands since German Physiologist Hans Berger in 1924?



Whole brain rs-MEG source-amplitude images averaged from 41healthy subjects in MNI-152 atlas coordinates from Fast-VESTAL in alpha (first row), beta (second row), gamma (third row), and low-frequency (delta plus theta, fourth row) bands.

Huang et al., NeuroImage, In press

Gamma-band hyper- and hypo-activities in PTSD vs healthy controls (t-test, corrected p<.01)

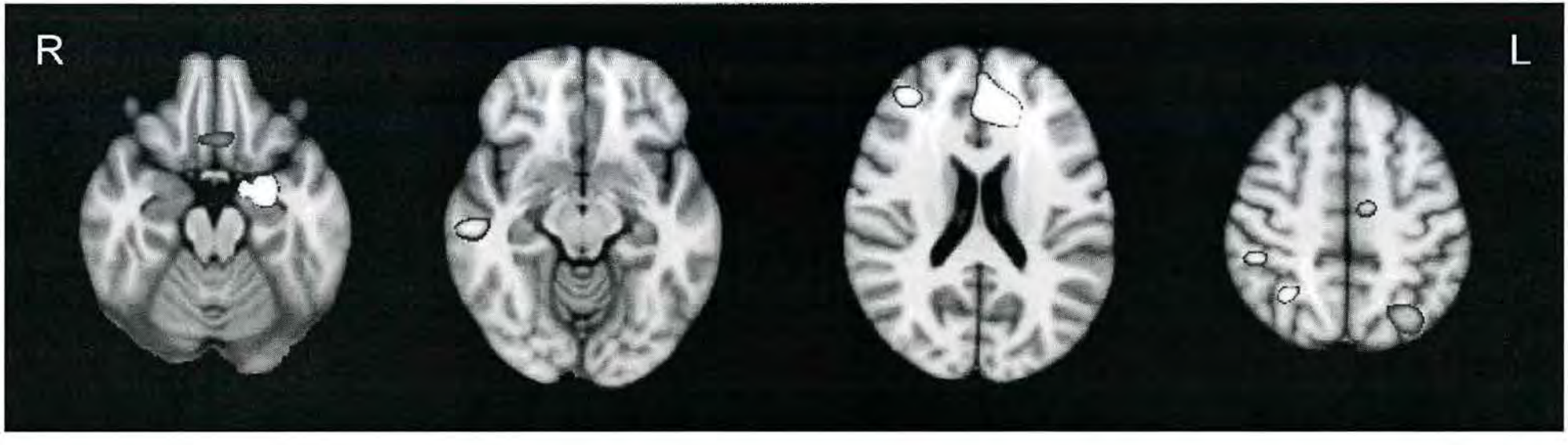


- Hyper-activity: L-R Amygdala; Anterior cingulate; R insular cortex; R frontal; R temporal lobe
- Hypo-activity: inferior medial frontal lobe

Developing MEG source imaging marker for PTSD: Study II

- Resting-state MEG recording with eyes-closed
- 38 EOD Marines with CAPS total: 0-75
- Compute correlation between MEG source imaging for gamma band activity (30-100 Hz) and CAPS total score

Positive and Negative correlations between MEG gamma-band activity with CAPS Total Score (corrected p<.01)



- Positive correlations: L Amygdala; Anterior cingulate; R frontal; R temporal lobe; R parietal areas
- Negative correlation: inferior medial frontal lobe; L parietal area

Summary: MEG Gamma-band Fear-Network imaging marker for PTSD

- In Veterans with PTSD, MEG gamma-band source imaging showed hyper-activity in amygdala, ACC, and other regions in fear network, but hypo-activity in inferior medial frontal lobe.
- In EOD Marines, MEG gamma-band activity in L amygdala, ACC, and other regions showed positive correlation with CAPS total score, but inferior medial frontal lobe showed negative correlation with CAPS total score.

Acknowledgement

- **Support**
  - Funding: VA Merit Review Grants (PI: Huang, Lee, Canive), Marine Resilience Study-II (PI: Baker, Exploratory Project PI: Huang), NFL (PIs: Huang, Lee), McDonnel Foundation via Brain Trauma Foundation (PI: Ghajar; site PIs: Lee, Huang)
  - Investigator Collaboration: VA San Diego Healthcare System, UCSD.

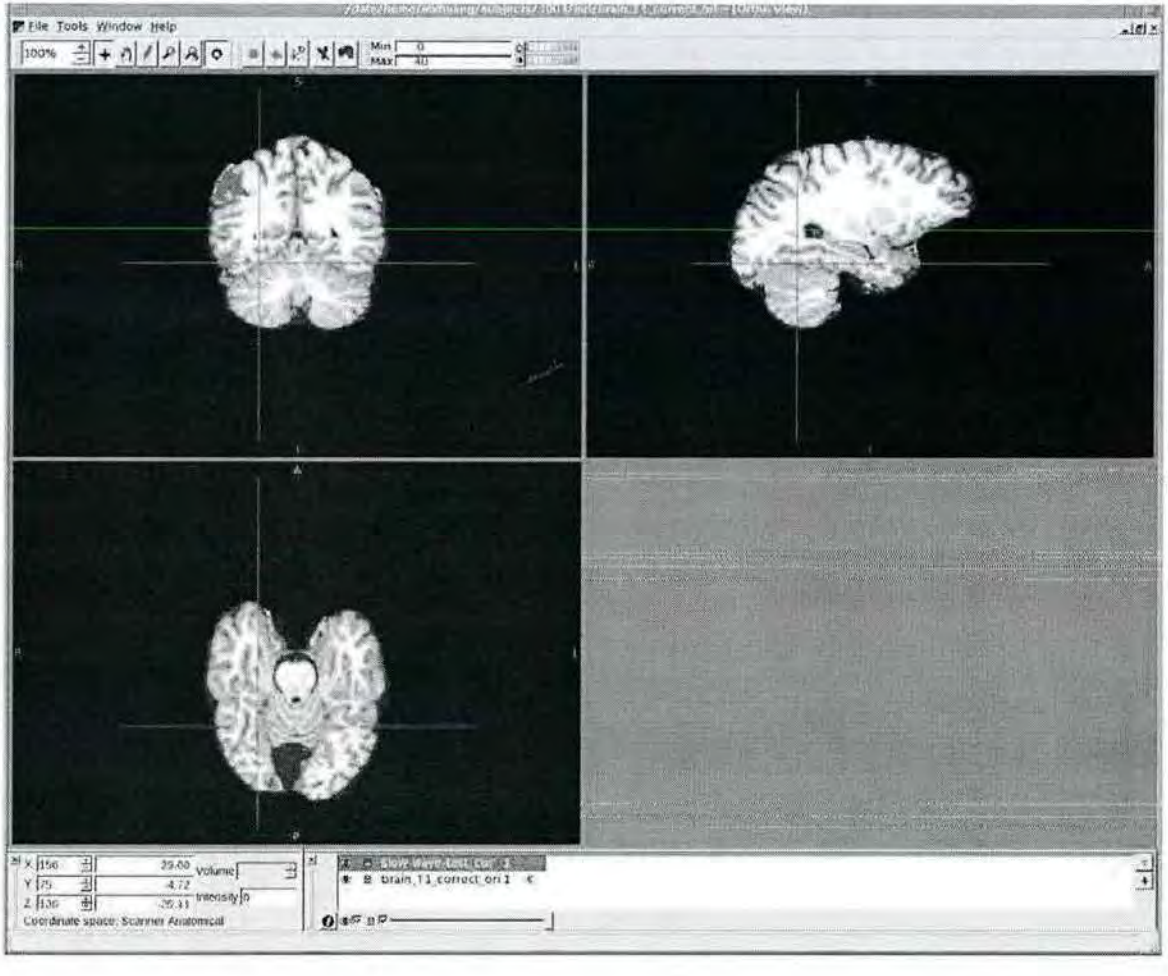
Dewleen Baker
Annemarie Angeles
Ashley Robb
Victoria Risbrough

Roland Lee
Sharon Nichols
Mithun Diwakar
Tao Song

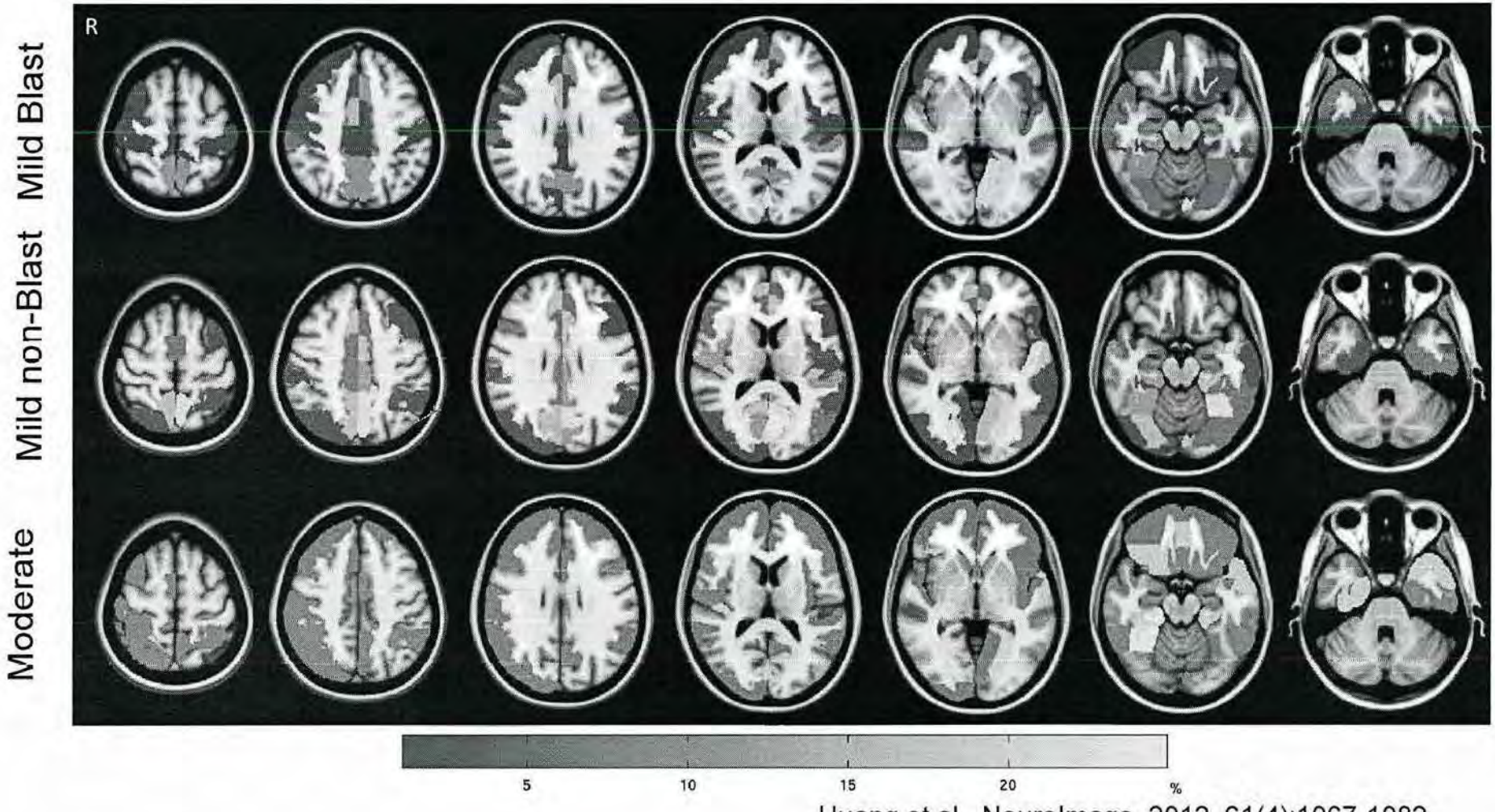
Invisible Injuries become not so Invisible

TV Interview with Col G.I. Wilson with KPBS:

http://www.youtube.com/watch?v=uhlANIGAJXA

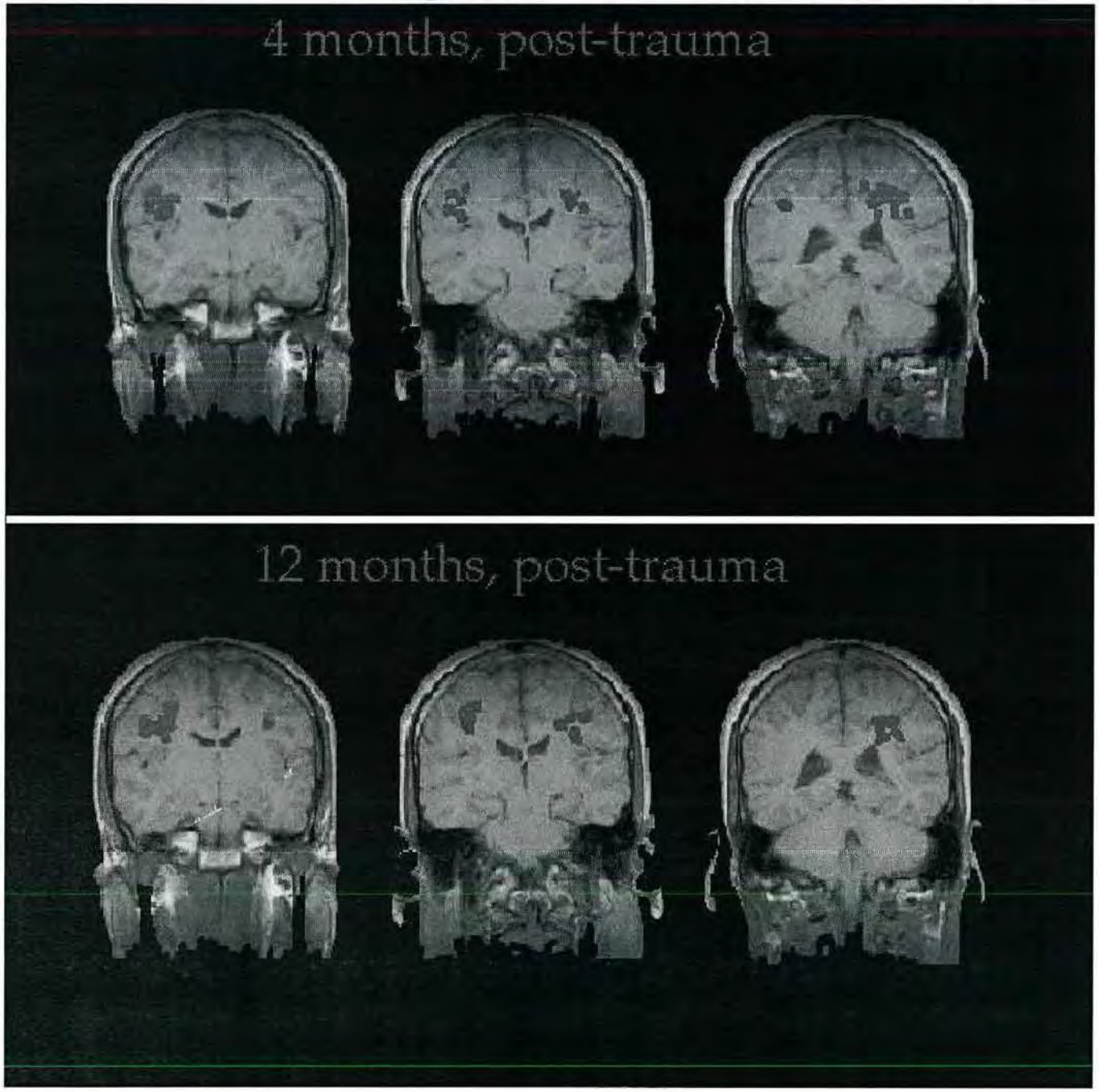


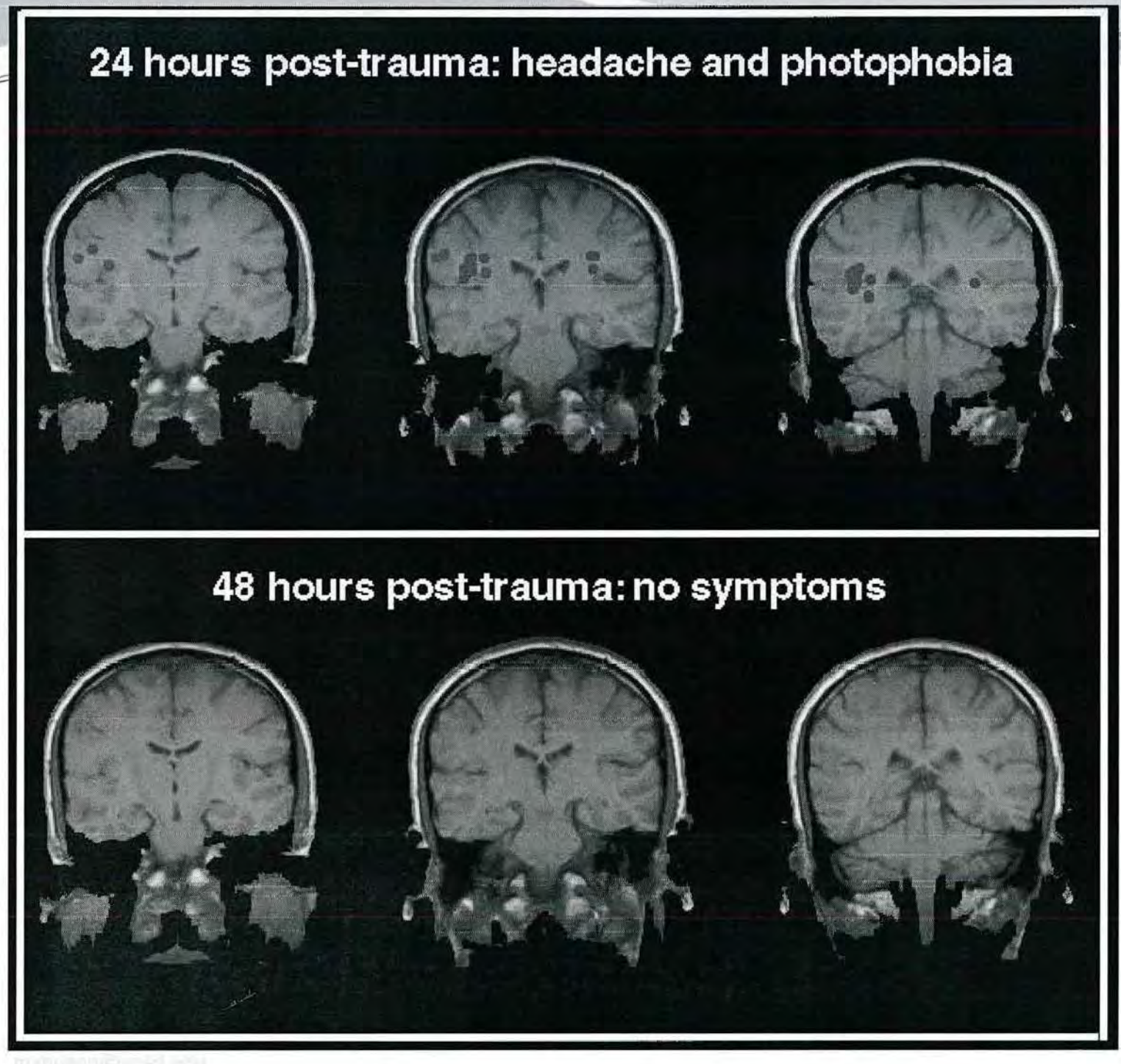
Percent-likelihood of MEG Slow-wave Generation across Brain regions



Huang et al., NeuroImage, 2012, 61(4):1067-1082.

Slowing in head trauma




24 hours post-trauma: headache and photophobia
48 hours post-trauma: no symptoms

MRS-II
The Marine Resilience Study II

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NextGen Metabolomics for Determining the Predeployment Risk and Postdeployment Diagnosis of PTSD and TBI

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University of California, San Diego School of Medicine, and
The Veterans Affairs Center for Excellence in Stress and Mental Health (CESAMH)

Name: Robert K. Naviaux, MD, PhD

Disclosure of financial relationship(s) with regard to:

Company *(Name, country)*	**Scientific research**	**Other:** (e.g. Advisory board, share holder)
Clinical Metabolomics (ClinMet), USA	None	Co-founder and stakeholder

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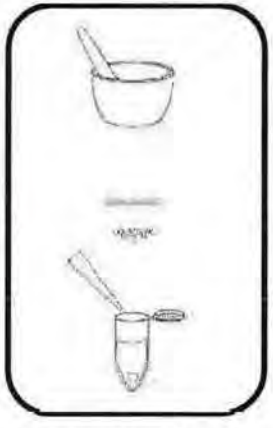
UCSD Metabolomics Overview

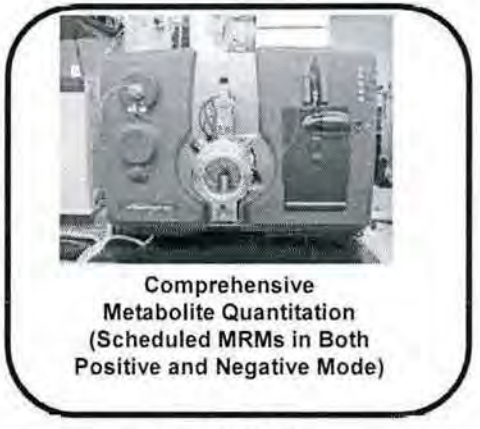
Blood / Urine / Tissue



Sample Prep

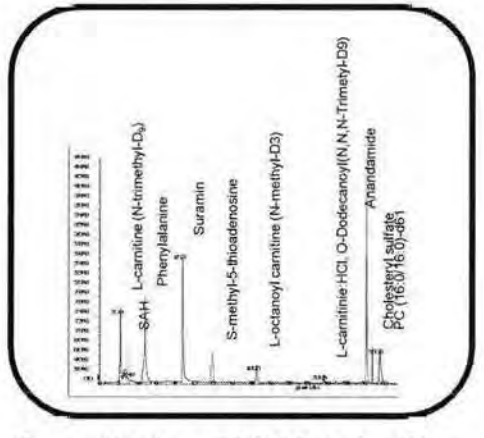
Polar &
Nonpolar
Metabolites
+
Internal
Standards

HPLC

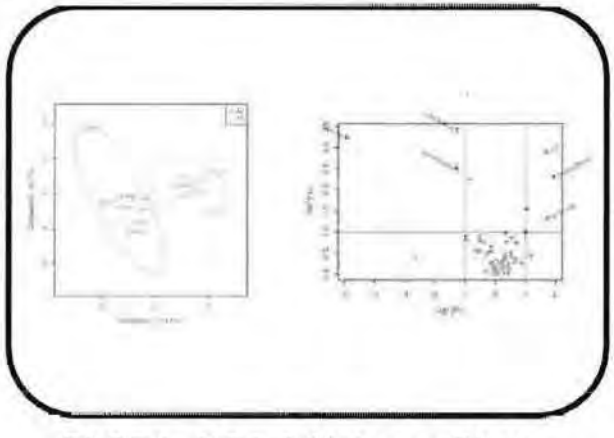


Comprehensive
Metabolite Quantitation
(Scheduled MRMs in Both
Positive and Negative Mode)

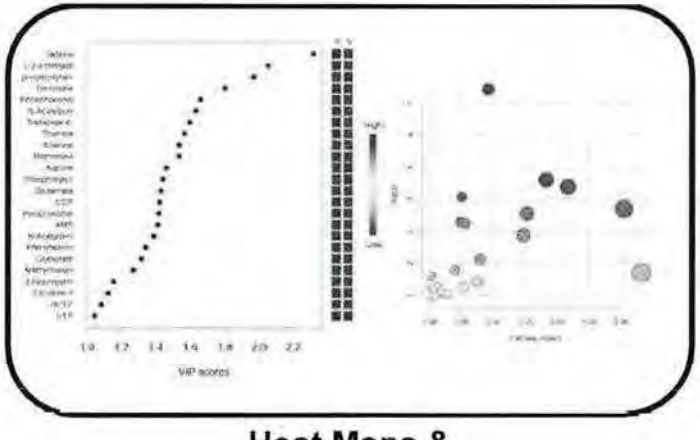
LC-MS/MS



Quantitate ~500 Metabolites



PLSDA, PCA & Volcano Plots



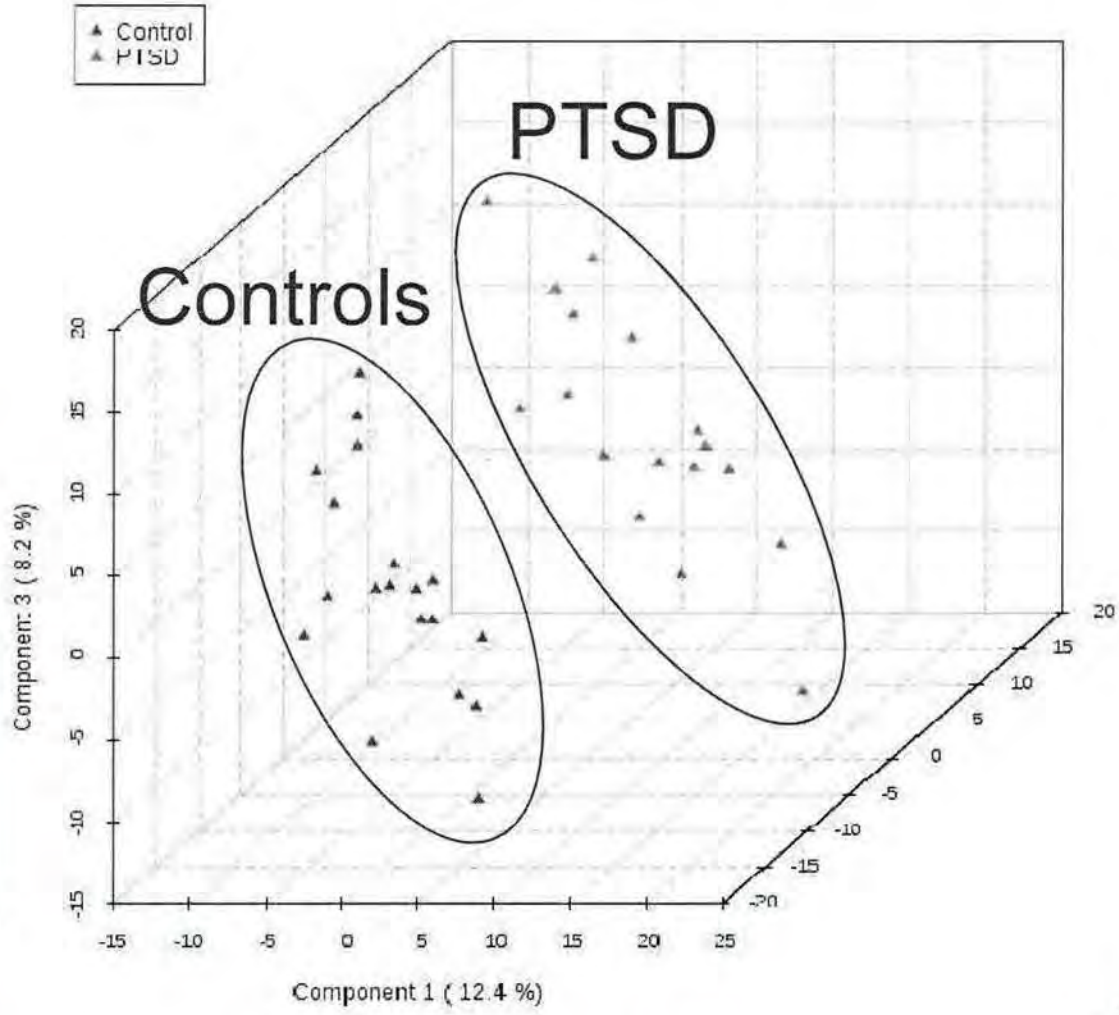
Heat Maps &
Biochemical Pathway Visualization

MRSII–Study design

Phenotype: based on CAPS

Metabolomic Diagnosis of PTSD—

Partial Least Squares Discriminant Analysis (PLSDA)



20 Metabolites were diagnostic

N = 19 Controls, 18 PTSD

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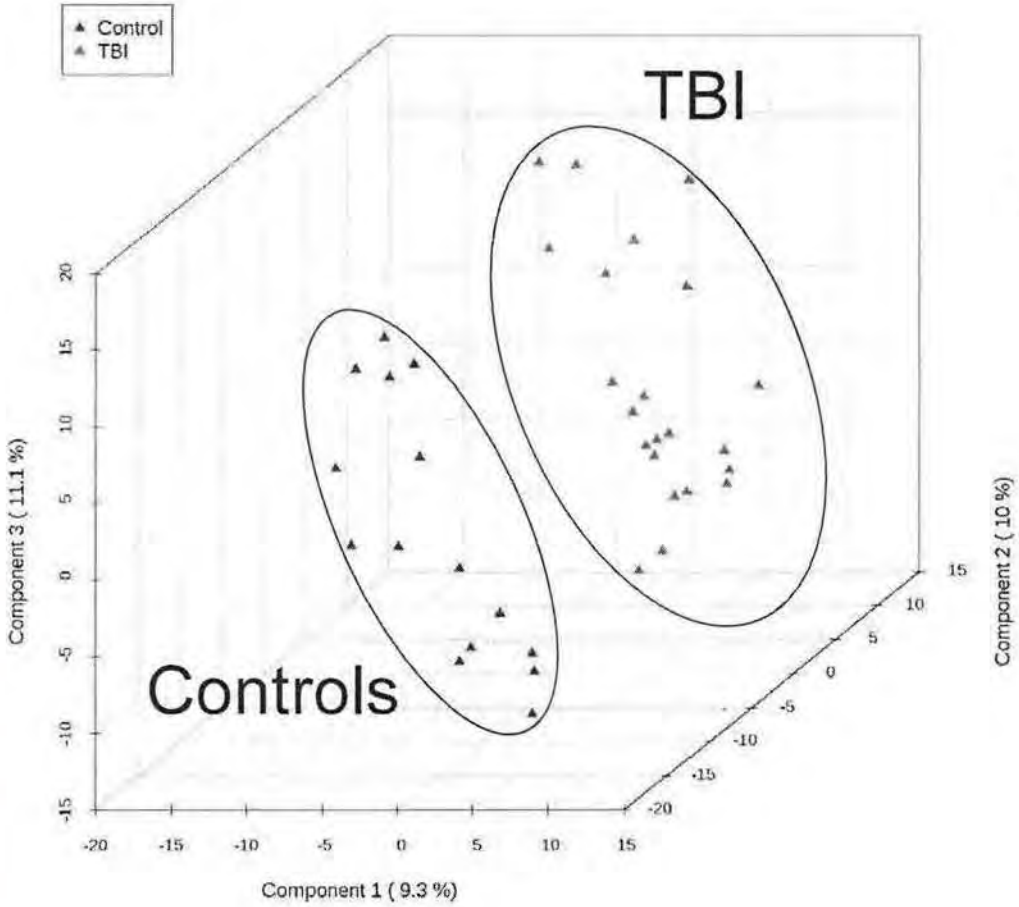
Metabolomic Diagnosis of TBI



24 Metabolites were diagnostic

N = 22 TBI, 16 Controls

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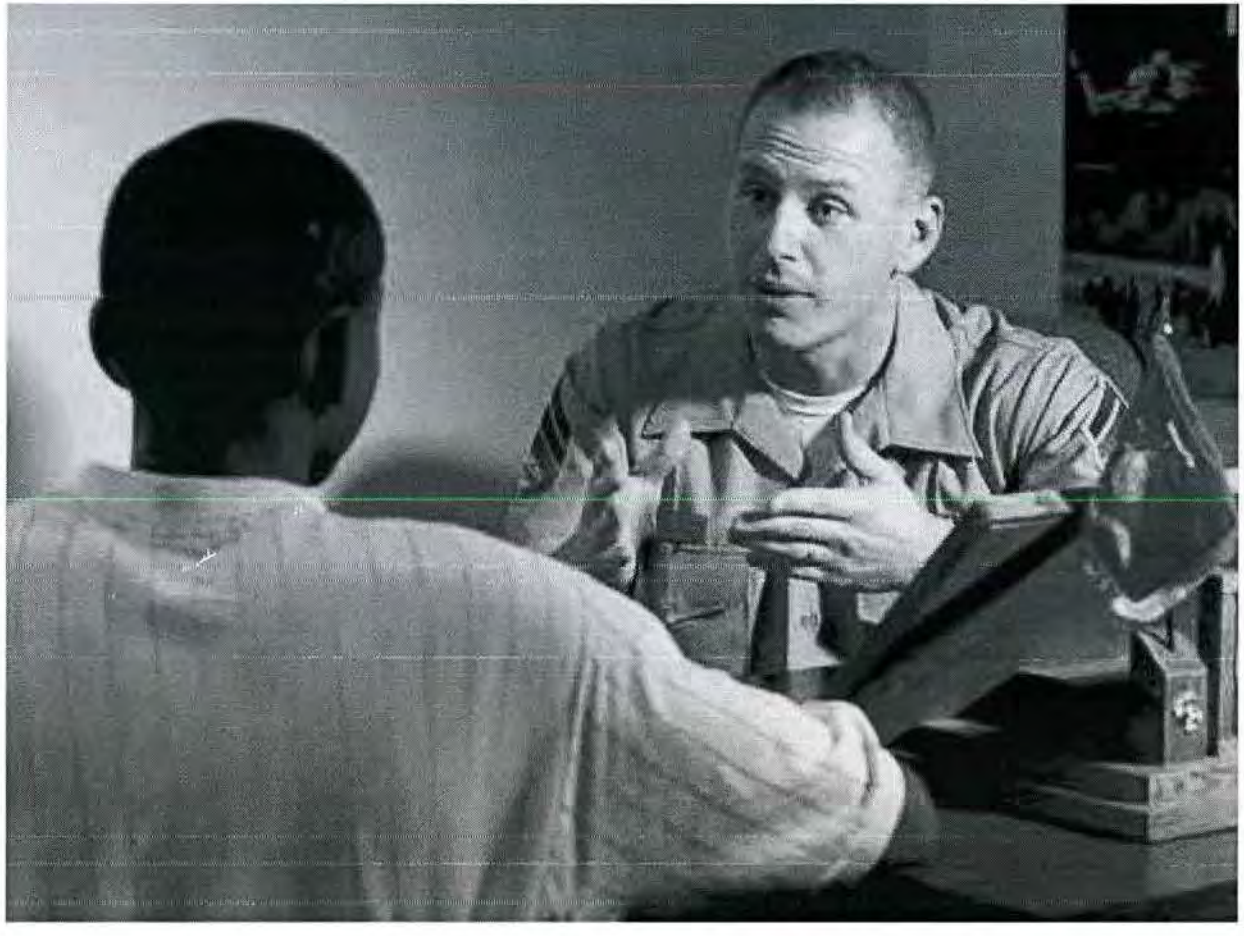

Can the risk of PTSD be measured before deployment?

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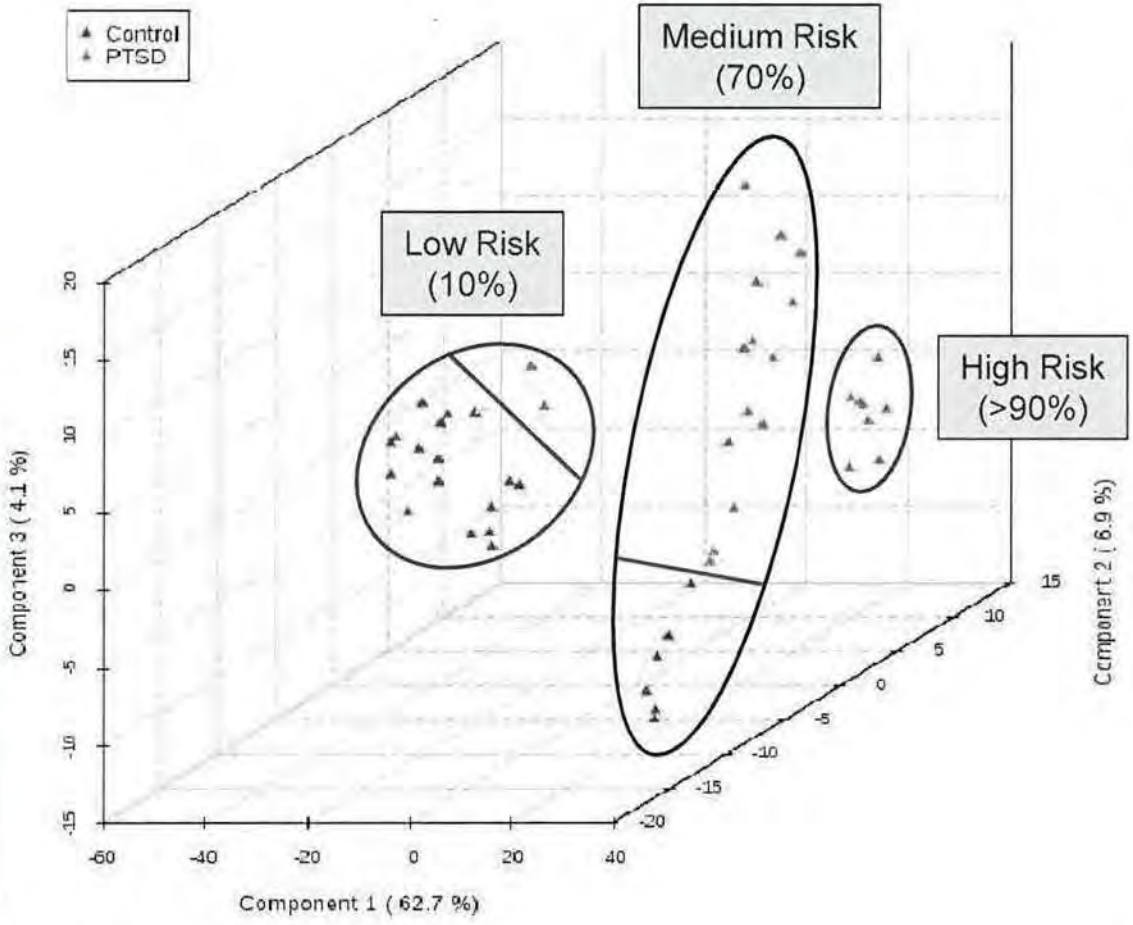
Metabolomic Assessment of PTSD Risk Before Deployment



30 Predeployment Metabolites were Predictive

N = 24 Controls, 24 PTSD

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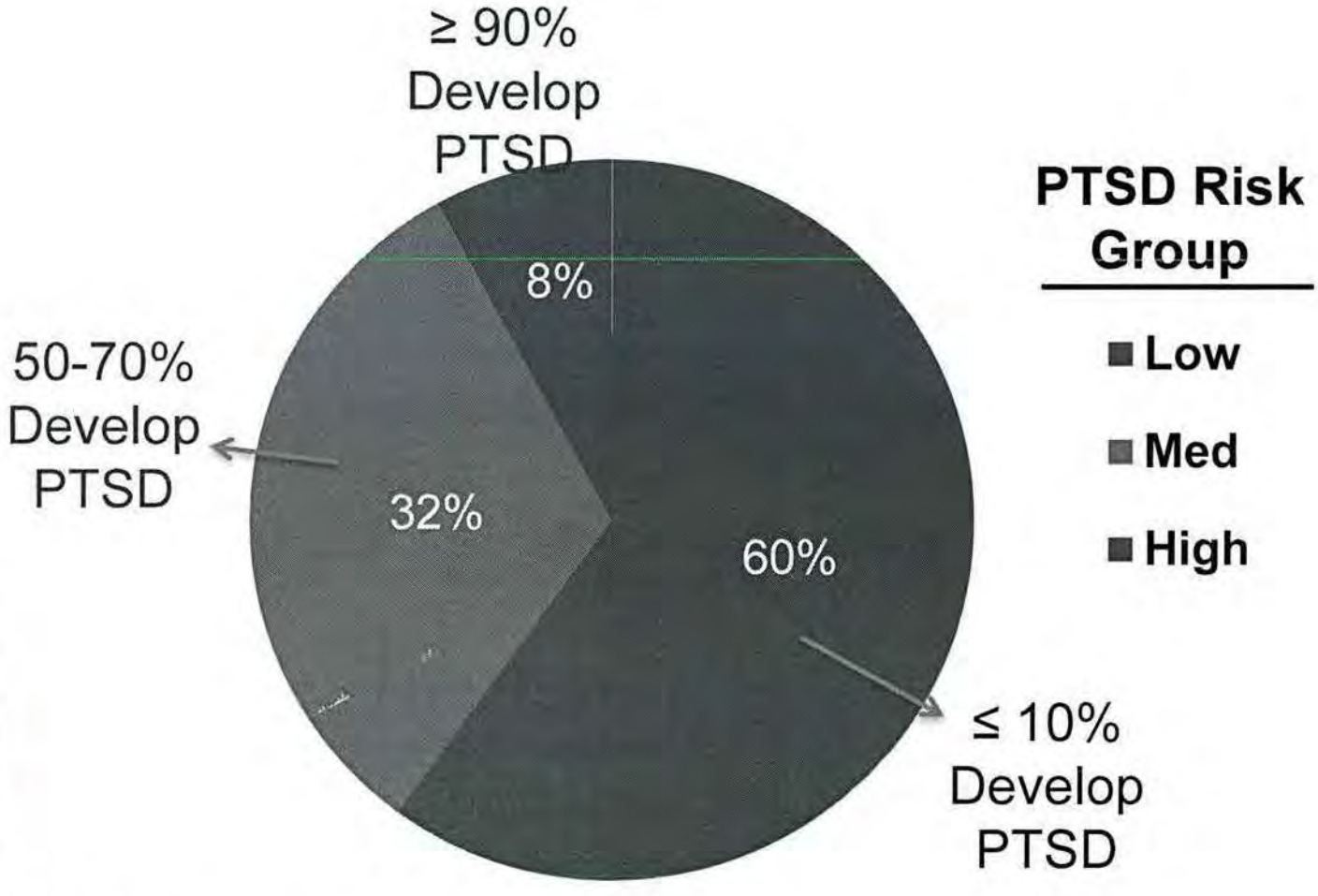
Metabolomic Risk Stratification for PTSD

(US Data for Deployment to Middle East Theaters*)



*Calculations based on an *a priori* risk of 20%

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Pathway Analysis

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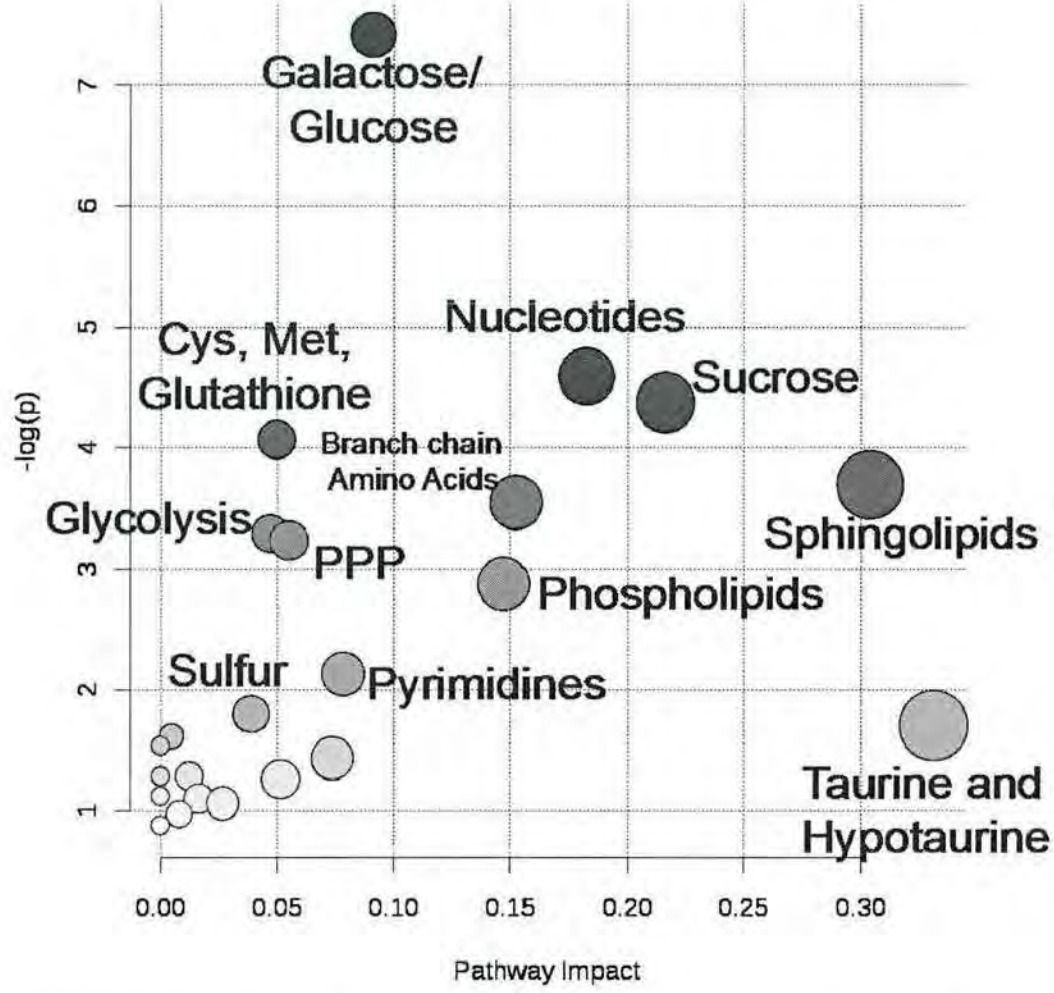
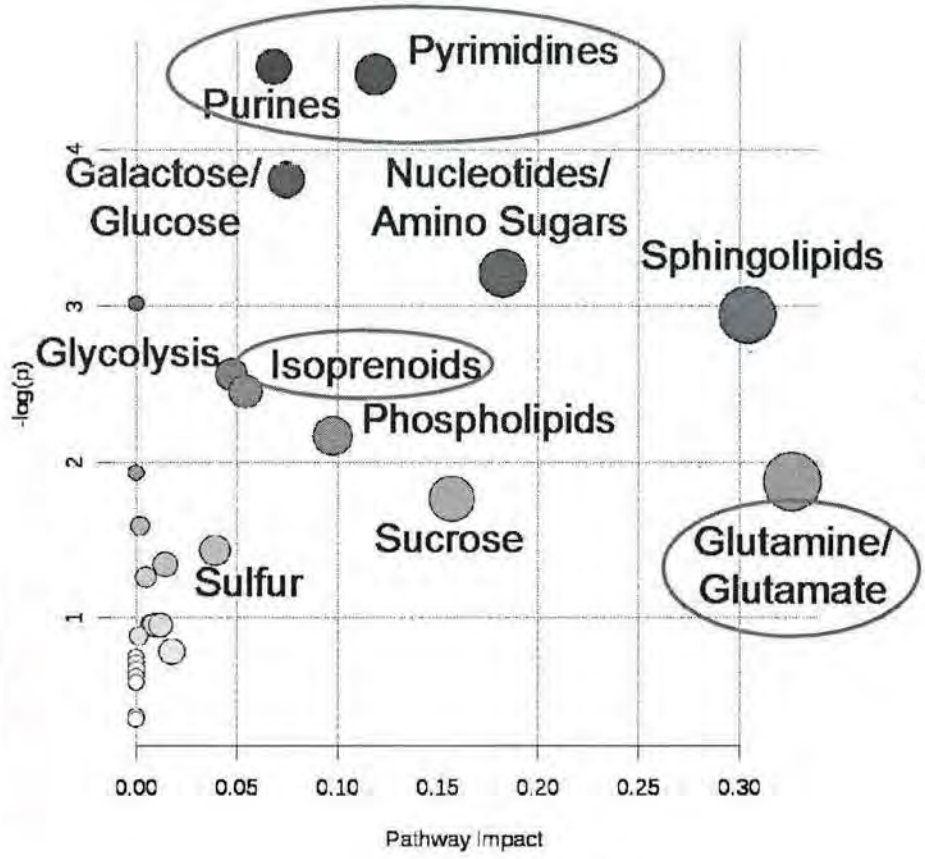
Metabolomic Features of PTSD– Pathway Enrichment Analysis



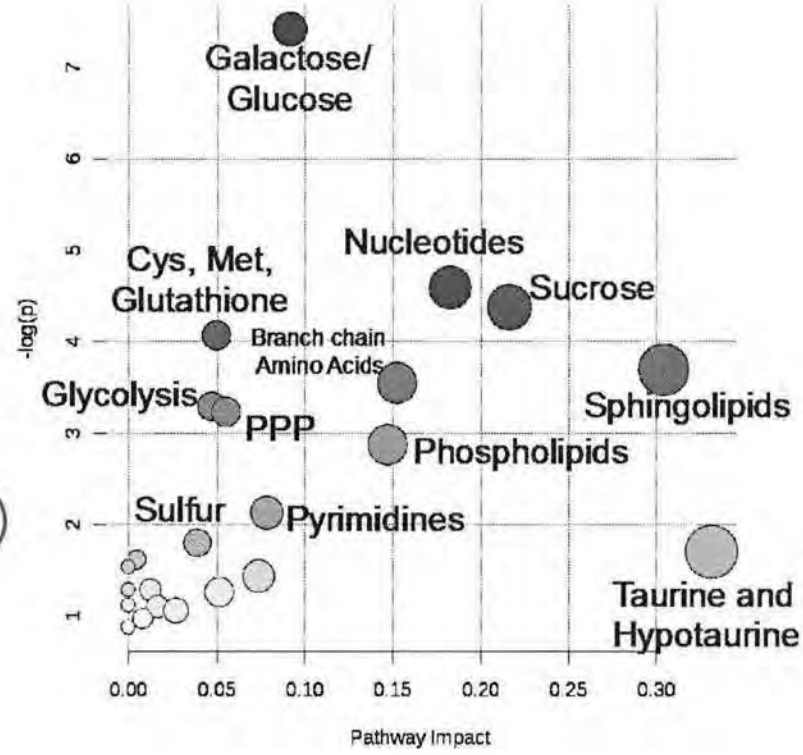
N = 19 Controls, 18 PTSD

Metabolomic Features of TBI– Pathway Enrichment Analysis

TBI

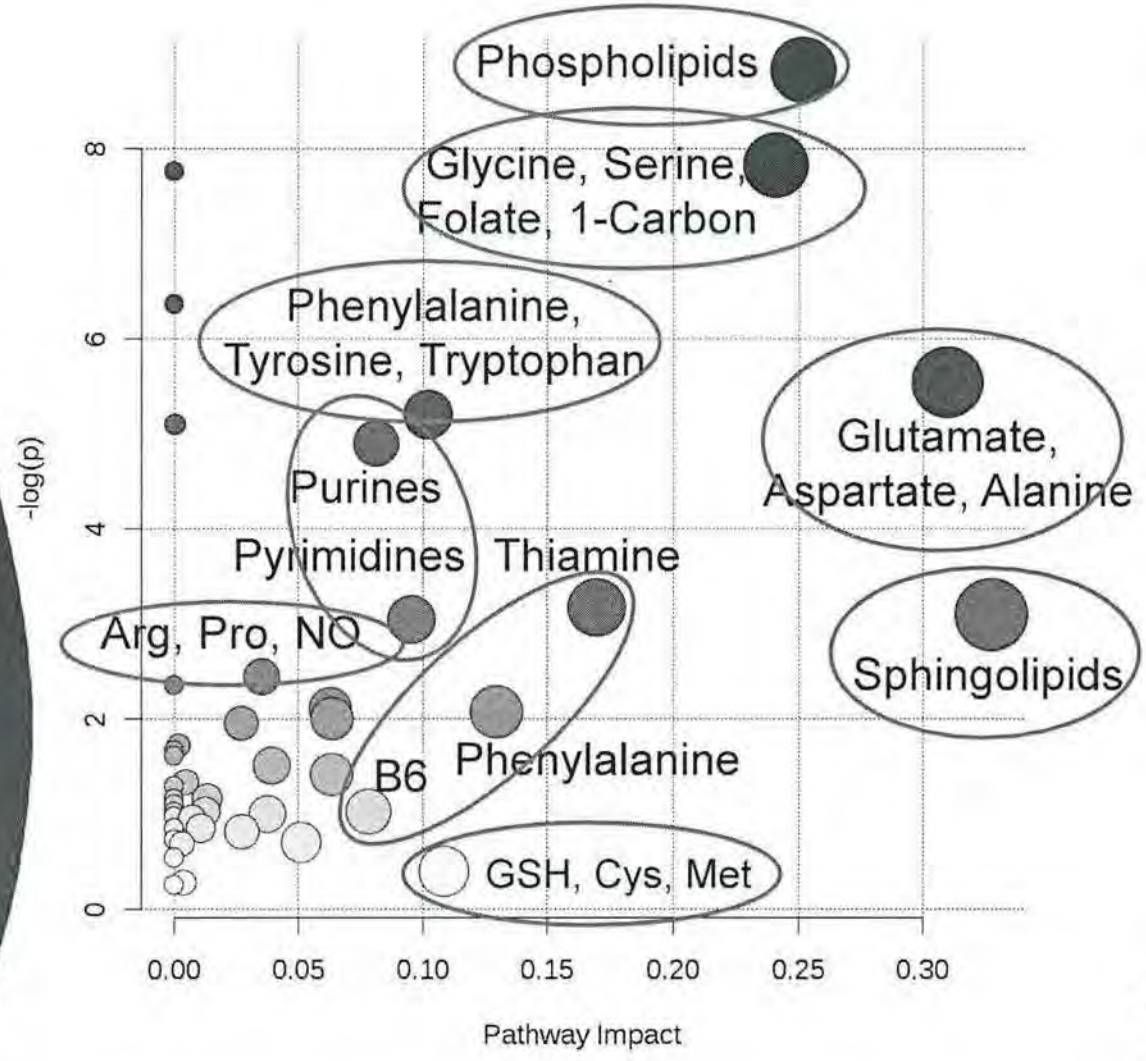


PTSD



N = 22 TBI, 16 Controls

Pathways Enriched in Predeployment Marines Who Later Develop PTSD



Predictive Pathways

1. Phospholipids and Sphingolipids
2. 1-Carbon Metabolism
   Formate, Glycine/Serine, methylation
3. Neurotransmitter synthesis
   Catacholamines, Serotonin, Glutamate, GABA
4. Purinergic Signaling
5. Urea/NO Cycle
6. Vitamin metabolism
   B6, Thiamine, Folate, B12
7. Glutathione, Cysteine, Methionine

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Conclusions—NextGen Metabolomics Interim Analysis of MRSII Samples

- **Diagnosis of PTSD**
  - » 20 metabolite biomarker signature
- **Diagnosis of TBI**
  - » 24 metabolite biomarker signature
- **Predeployment Risk of PTSD**
  - » 30 metabolites biomarker signature predicts risk
  - » Stratifies Marines into Low, Medium, and High-risk groups
    - Low: ≤ 10%
    - Medium: 50-70%
    - High: ≥ 90%

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Collaborators and Support

MRS-II

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The MRS Research Team

DOD Funding

Marine Corps
Navy BUMED

Naviaux Lab

Kefeng Li, PhD

Jane Naviaux, MD, PhD

Lin Wang, MD, PhD

Thank You

- MRSII Demonstration Grant Program
- Jane Botsford Johnson Foundation
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- The UCSD Christini Fund
- Wright Family Foundation
- Lennox Foundation

Christine Shimizu
1996-1998

Special Thanks to the Marine and Sailor Volunteers of the Marine Resiliency Study

Marine Resiliency Study

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CRP as Predictor of PTSD Risk

Dewleen G. Baker M.D.

Professor
Department of Psychiatry
University of California San Diego
Director of Research
VA Center of Excellence for Stress and Mental Health

Inflammation

- Inflammation is associated with:
  - Metabolic syndrome
  - Atherosclerotic cardiovascular disease
  - Depression
  - Decreased Heart rate variability

- PTSD is associated with:
  - Metabolic syndrome
  - Atherosclerotic cardiovascular disease
  - Depression
  - Decreased Heart rate variability

Trauma and Inflammation

- **Studies support an association between trauma exposure with increased peripheral inflammation**

- O'Donovan A, Neylan TC, Metzler T, Cohen BE (2012)
  - Prospective study of patients with stable CVD (n=979)
  - Inflammation was indexed by a composite score incorporating the inflammatory markers interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP) and resistin.
  - Higher trauma exposure was associated with elevated inflammation at baseline (β=.09, p=.01) and at five-year follow-up (β=.09, p=.03).

PTSD and Inflammation

- **Observational studies largely support an association of post-traumatic stress disorder with increased peripheral inflammation**

- Spitzer C, Barnow S, Völzke H, et al (2010)
  - Cross sectional study; 3049 adults living in the community
  - PTSD positive participants had significantly higher odds for elevated CRP values than those without PTSD (OR=2.27; 95% CI: 1.32-3.93).

- Heath NM, Chesney SA, Gerhart JI, et al (2013)
  - Cross sectional study; 139 urban women who have a high likelihood for having experienced interpersonal violence
  - Individuals who experience clinical levels of PTSD exhibited higher CRP levels, and this relationship held after adjusting for comorbid depression

PTSD and Inflammation

- **Observational studies largely support an association of post-traumatic stress disorder with increased peripheral inflammation**

- Plantinga L, Bremner JD, Miller AH, et al (2013)
  - 238 male middle-aged military veteran twin pairs (476 individuals), selected from the Vietnam Era Twins Registry
  - 12.4% of participants had a lifetime history of PTSD
  - Assessed inflammation using multiple measures including levels of high-sensitivity C-reactive protein (hsCRP)
  - Elevated hsCRP associated with PTSD, and the association may be confounded by shared non-genetic, antecedent familial and environmental factors

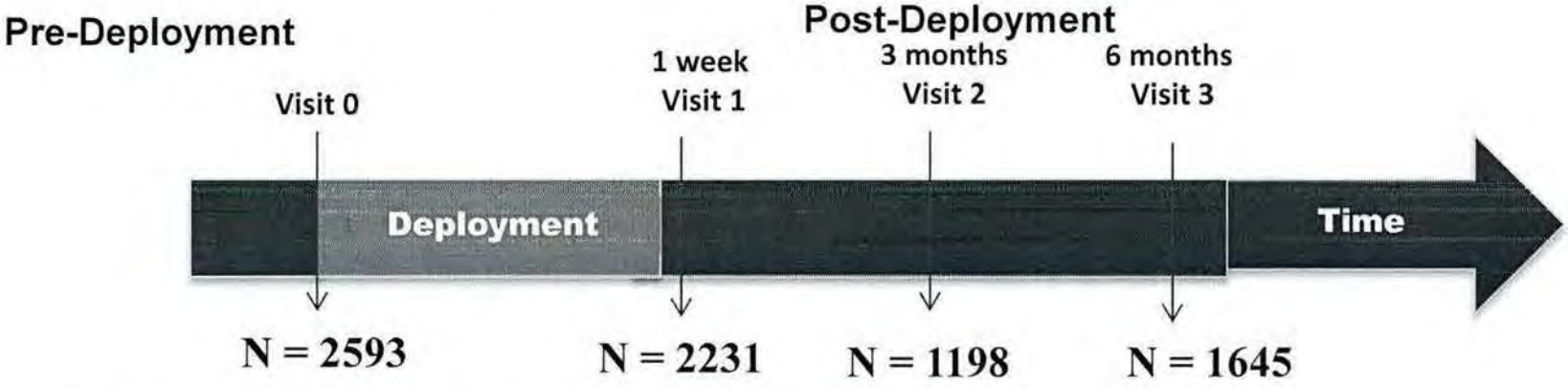
Translational Study: PTSD and Inflammation

- **Findings suggest possible functional relevance of TLR9 in protecting stressed mammals from overreacting to traumatic experiences**

- Zimmerman G, Shaltiel G, Barbash S, et al (2012)
  - Cross sectional study; 37 PTSD/37 Control Subjects: Association of serum interleukin-1β increases with symptoms severity and volumetric brain changes in post-traumatic stress disorder patients
  - Manipulated both inflammation and anxiety in predator scent-stressed mice by peripheral administration of both mEN101 and BL-7040, which emerged as selective TLR9-mediating activators of an alternative NFkB pathway
  - Suggest that moderate activation of TLR9 suppresses peripheral levels of brain penetrating cytokines and minimizes the behavioral consequences of acute stress exposure

Marine Resiliency Study

- Marine Resiliency Study (MRS) is a prospective (from 2008) field study of approximately 2,600 Marines and Sailors deployed to Iraq or Afghanistan



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MRS Data Sources

Psychological assessments

Historical
- Self-report questionnaires

Neuropsychological
- Neurocognitive performance

Psychiatric and medical
- Clinical interviews

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Military archives
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MRS Secure database

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Biological samples
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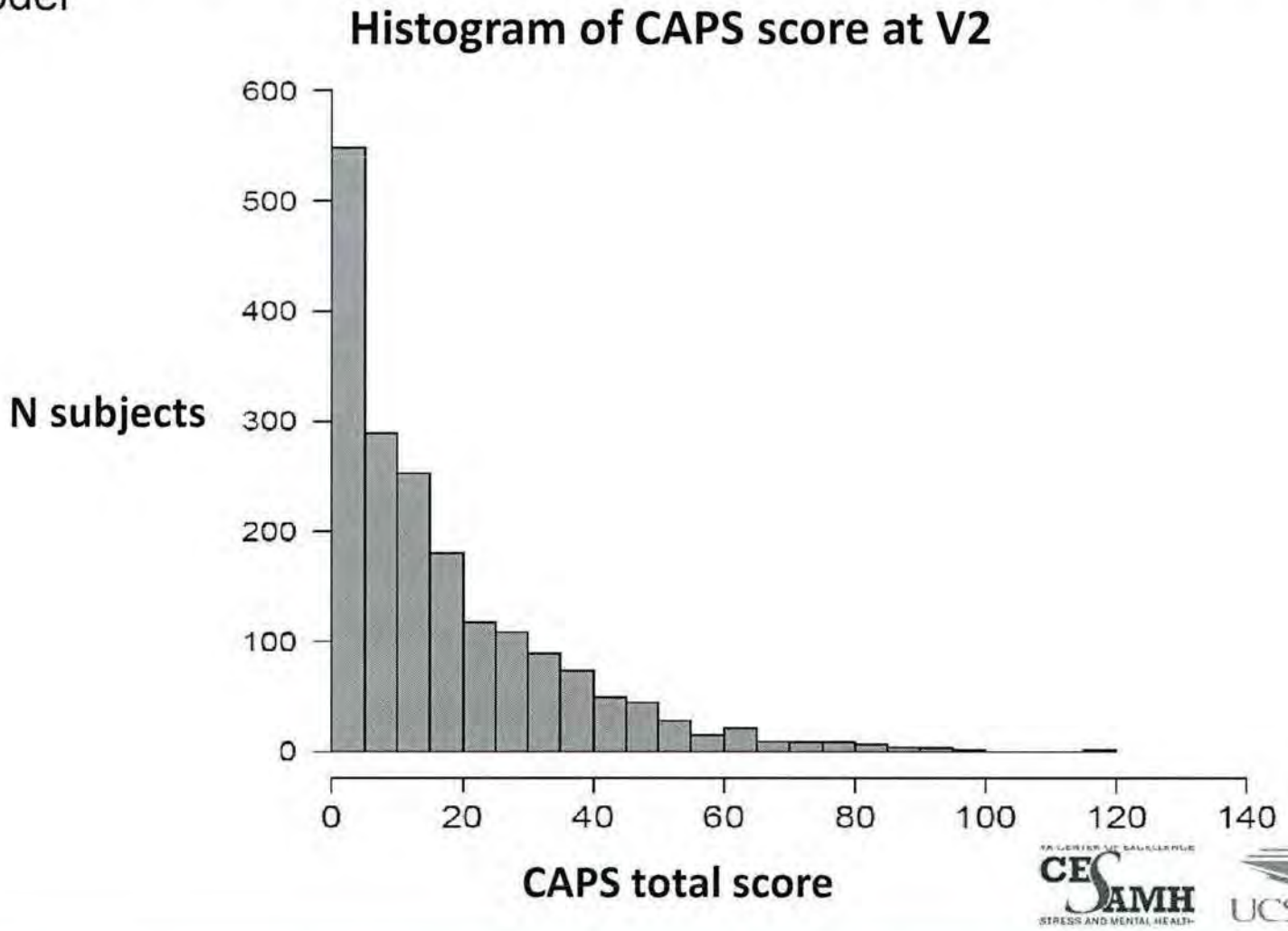
Psychophysiology
- Startle reactivity, Fear learning and extinction

Genomics

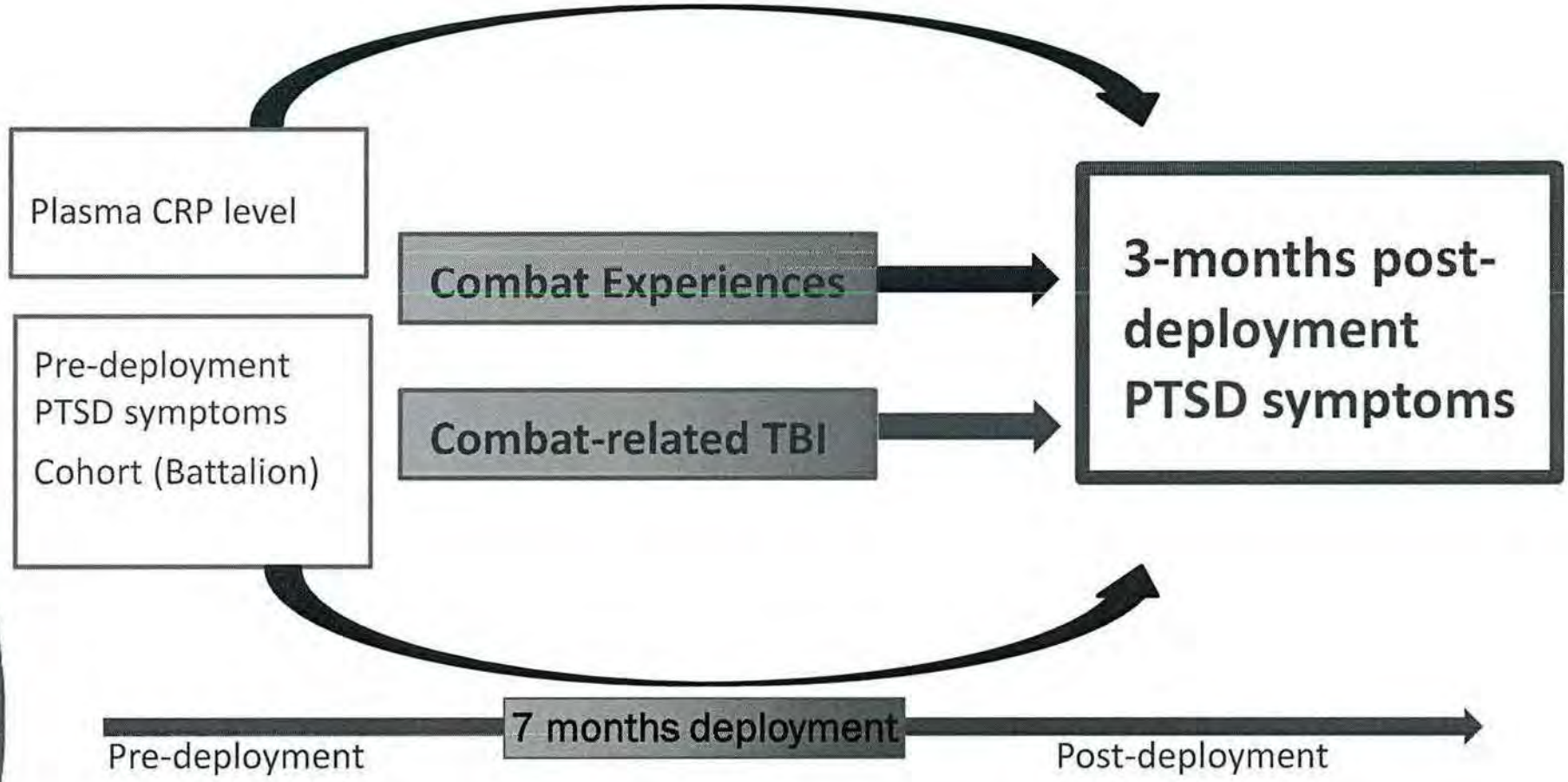
- **GWAS (complete data)**
- **Methylome (subset)**
- **Epigenome (subset)**

ZINB Distribution

- CAPS total score is not a normally distributed trait in MRS. The trait cannot simply be transformed to normality because there are too many zero value scores
- Zero inflated negative binomial (ZINB) distribution: ZINBR best statistical model



Is a proxy for inflammation (CRP) a Risk Factor for PTSD? A Prospective Analysis



Participants: Baseline plasma CRP concentrations were included from 2,484 subjects (97.2%) and baseline CAPS score from 2,533 (99.1%)

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Is a proxy for inflammation (CRP) a Risk Factor for PTSD? A Prospective Analysis

- Additional considerations
  - Potential confounders were selected for inclusion in regression modeling on the basis of their univariate association, at a lenient significance threshold of $p<0.2$, with both the outcome (post-deployment CAPS), & predictor of interest (plasma CRP concentration).
  - Potential confounders were selected for inclusion in regression modeling on the basis of their univariate association, at a lenient significance threshold of $p<0.2$
  - Confounders considered: Depression (BDI-II), Anxiety (BAI), Alcohol Use (AUDIT), Tobacco Use, Body Habitus: baseline waist circumference, body mass index (BMI), and blood pressure (BMI), baseline military characteristics (history of prior deployment, duration of service, rank) and demographics (age, race, education, marital status)
  - None of these potential confounders met criteria for inclusion in regression models

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ZINB model results

CAPS at visit 2 as predicted under a ZINB model (Eraly et al., under review)

	Zero model			Count model			
Parameter	**OR**	**95% CI**	**P value**	**Fold-change**	**95% CI**	**P value**	**Overall P value**
Intercept	0.879	1.527 - 3.800	0	10.892	9.747 - 12.171	0	0
Cohort 1							
Cohort 2	0.296	0.183 - 0.479	0.016	0.915	0.809 - 1.035	0.354	0
Cohort 3	0.329	0.195 - 0.555	0.035	0.755	0.662 - 0.861	0	0
Cohort 4	0.52	0.292 - 0.928	0	0.97	0.843 - 1.117	0	0
CAPS0	1.097	1.075 - 1.119	0	1.02	1.018 -1.023	0	0
CES	1.029	1.005 -1.054	0.019	1.009	1.004 -1.014	0.001	0
PBE	1.077	1.026 -1.131	0	1.039	1.026 -1.053	0	0
log CRP	1.507	1.153 -1.969	0.003	1.062	0.991 -1.138	0.086	0.002

- For each predictor variable we obtain two sets of coefficients: One for it's prediction of zero and another for it's prediction of count
- CRP was not significantly correlated with baseline CAPS, BDI, or BAI scores (the Spearman correlation coefficients and corresponding p values were, respectively: 0.012, p= 0.536; 0.001, p= 0.954; and -0.001, p= 0.973), indicating that CRP it is not a mediator or proxy for the effects of one of these other predictors on CAPS

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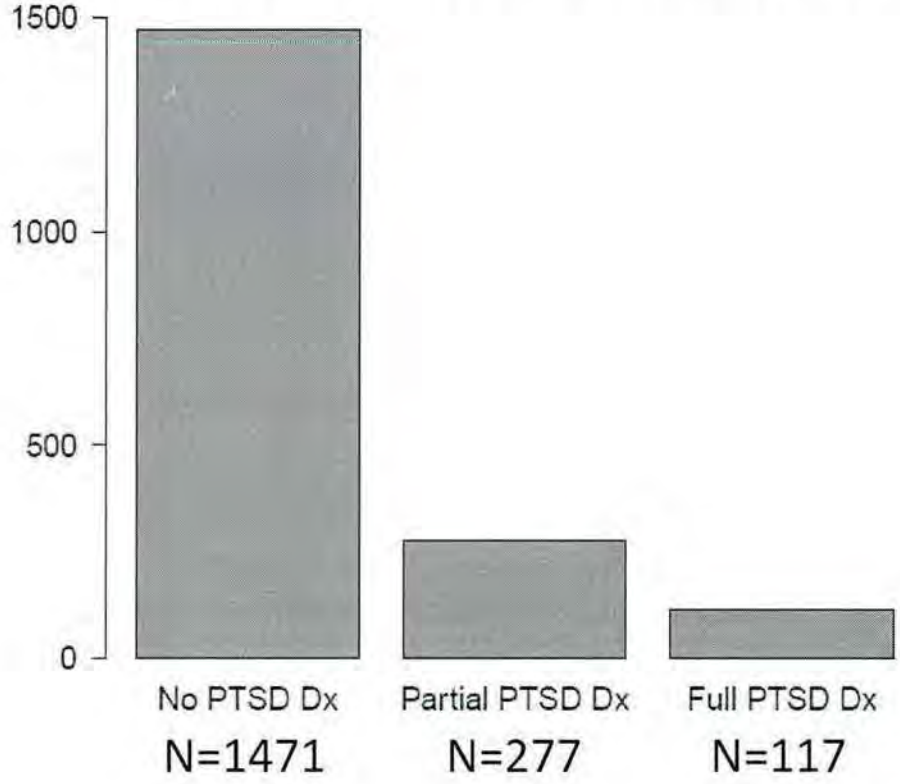
Grouping subjects using CAPS DSM-IV diagnosis:

- As an alternative to modeling based upon raw score, we instead used diagnosis to group subjects and use ordered logistic regression to model the data
- There are 3 groups in order of severity: No diagnosis, partial PTSD diagnosis (stringent or lenient criteria), or the DSM-IV based PTSD diagnosis

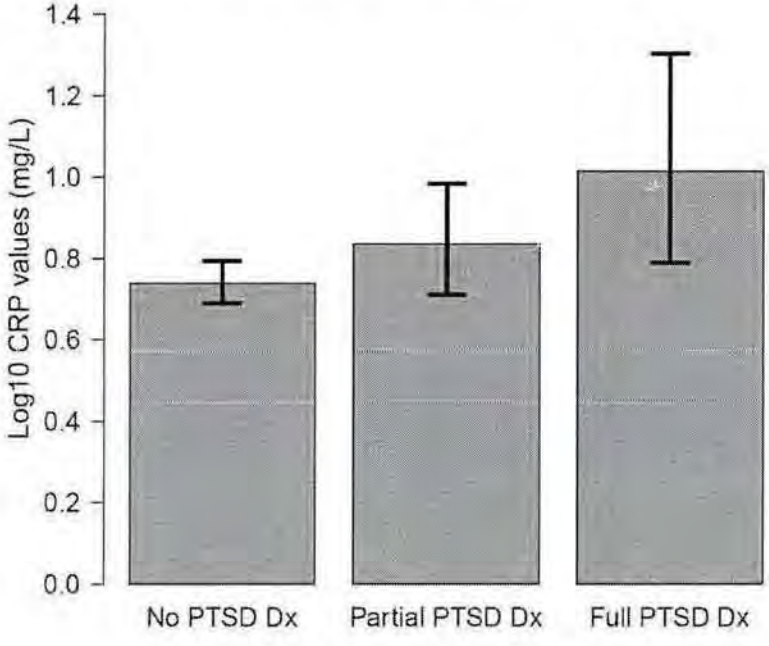
Quantity of subjects within each CAPS group at V2



Is a proxy for inflammation (CRP) a Risk Factor for PTSD? A Prospective Analysis

Using the ordered logistic model gives us a similar inference to the ZINBR

Mean (+/- 2 SEM) adjusted values of CRP by CAPS group



CAPS at visit 2 as predicted under an ordered logistic model

Parameter	OR	95% CI	P
Cohort 2	0.846	0.537 - 1.347	0.475
Cohort 3	0.514	0.314 - 0.845	0.008
Cohort 4	0.923	0.552 - 1.553	0.762
CAPS0	1.053	1.045 - 1.062	0
CES	1.035	1.019 - 1.051	0
PBE	1.103	1.058 - 1.151	0
log CRP	1.3	1.026 - 1.646	0.029

Conclusions

- **Findings suggest that levels of this inflammatory marker may predict resilience versus risk for PTSD symptom emergence, and thus could have implications for preventative interventions**
- We report a significant effect of baseline CRP on post-deployment PTSD symptom emergence in Marine and Navy combatants
- CRP was not correlated with baseline measures of depression, anxiety, or PTSD, indicating that it is not a mediator or proxy for one of these other predictors
- In this study, CRP predominantly influenced the likelihood of subjects endorsing the presence vs. absence of PTSD symptoms rather than the extent of symptoms

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Acknowledgements

Marine Resiliency Study

- Mark Geyer Ph.D., Co-I, UCSD
- Paul Hammer M.D., Co-I, DCoE
- Gerald Larson Ph.D., Co-I, NHRC
- Brett Litz Ph.D., Co-PI, Boston VA/BU
- Jennifer Webb-Murphy, Co-I, NCCOSC
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- Nicholas Schork Ph.D., Co-I, Scripps Institute
- Jennifer Vasterling, Ph.D., Co-I, Boston VA/BU
- MRS Research Team

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- **Agorastos Agorastos M.D.,**
- **Nilima Biswas Ph.D., UCSD**

Statistics

- **Adam Maihofer M.S., UCSD**
- **Don Barkauskas Ph.D. USC**

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- **Marine Corps**
- **Navy Bureau of Medicine**
- **VA Health Services Research and Development**

Data Collection Assistance

- **CESAMH**
- **NC COSC, San Diego**

Acknowledgements

Special Thanks to the Marine and Sailor Volunteers of the Marine Resiliency Study

Thanks also to CESAMH and to NC COSC for Assistance in Data Collection

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Marine Resiliency Study

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Military Biomarker

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Department of Psychiatry
University of California, San Diego
Director of Research
VA Center of Excellence for Stress and Mental Health

MRS & MRS-II

- Marine Resiliency Study (MRS) is a prospective (since 2008) field study of Marines and Sailors deployed to Iraq or Afghanistan
- Established with support of Marine Leadership (HQMC)
- Study of predictors of risk and resilience predictors in PTSD
- Currently the only American military project that fully conforms to Institute of Medicine Guidelines for combat stress studies

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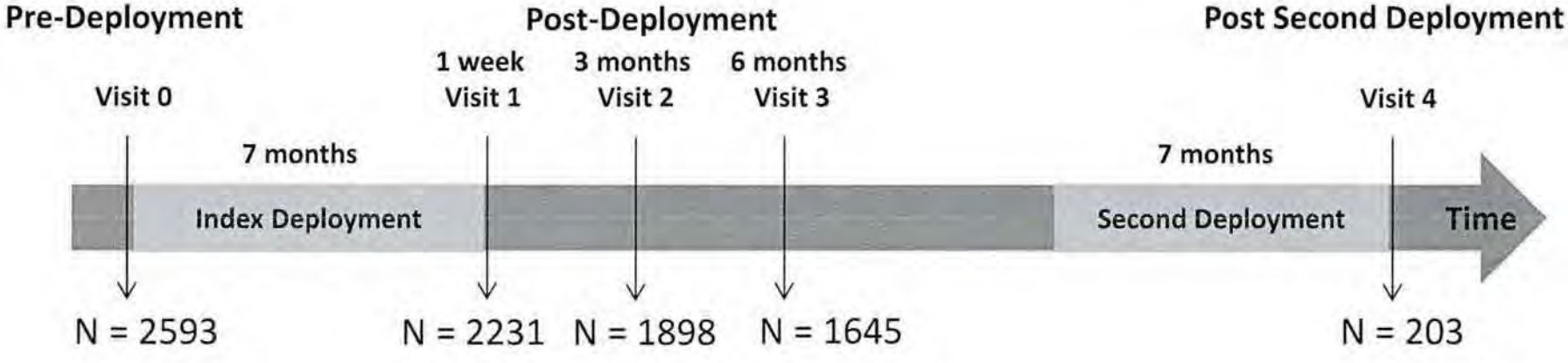
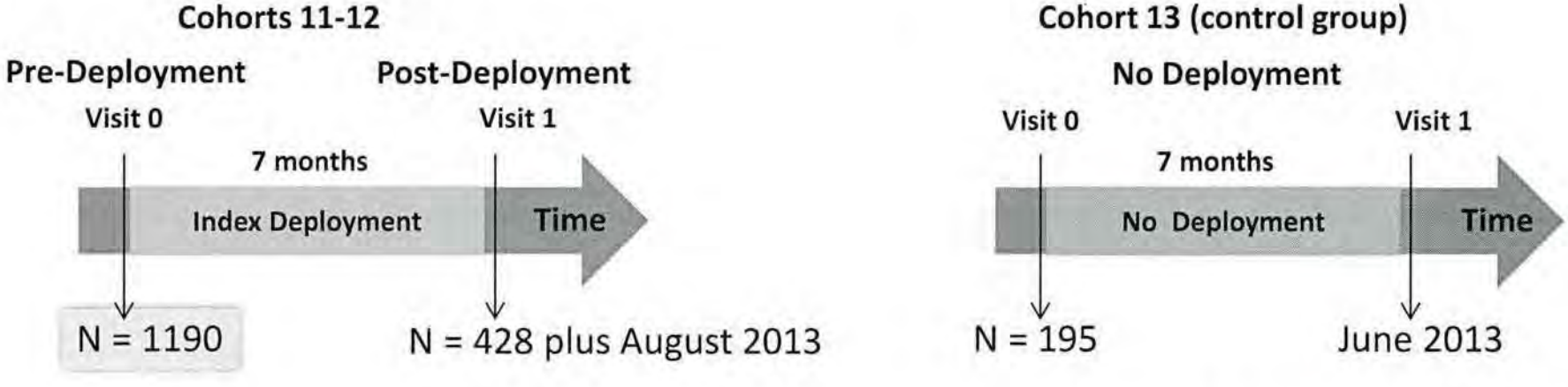
Marine Resiliency Study & MRS-II

MRS: 2008 – 2011 Cohorts 1-4 Data collection complete



MRSII: 2011 – 2013 (PIs Baker, Risbrough, Geyer) Cohorts 11-13



Timeline and Enrollment

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Psychophysiology
- Startle reactivity, Fear learning and extinction

Genomics

- **GWAS (complete data)**
- **Methylome (subset)**
- **Epigenome (subset)**

MRSII 2-corner subject selection criteria

Both cases and controls had to meet the following:

Had to have "use visit" criteria 1 (index deployment 1; refer to 'Grievance list').
Had to do the CAPS at predeployment visit (V0).
No CAPS DSMIV PTSD Diagnosis at predeployment visit.
No CAPS partial PTSD stringent diagnosis at predeployment visit.
CAPS score 39 or lower at predeployment visit.

Cases additionally had to meet the following:

Cases had to meet at least one of the following (item 1 or 2):
CAPS DSMIV PTSD diagnosis at V2 and/or V3,
CAPS partial PTSD stringent diagnosis with CAPS score above 39 at V2 and/or V3.

With the exception of 'Ming' or 'demo 2' project subjects, who if in absence of meeting either of the above, needed to meet at least one of the following (item i or ii):

CAPS partial PTSD stringent diagnosis.
CAPS partial PTSD lenient diagnosis and CAPS score above 39 at V2 and/or V3. (n=16 subjects added).

177 cases meet these criteria.

Controls additionally had to meet the following:

Controls had to meet all of the following items (1 to 4)
No CAPS DSMIV or partial PTSD stringent diagnosis at any additional timepoint (V2,V3)
No CAPS score above 39 at any visit.
CAPS done at all visits (V0,V2,V3)

With the exception of 'Ming' control subjects, who only needed at least one post deployment CAPS (n = 21 added).

CES >= 20 and/or PBE >= 8.

With the exception of 'Ming' control subjects, who were kept even if they did not meet this particular cutoff (n= 15 added).

346 controls meet these criteria.

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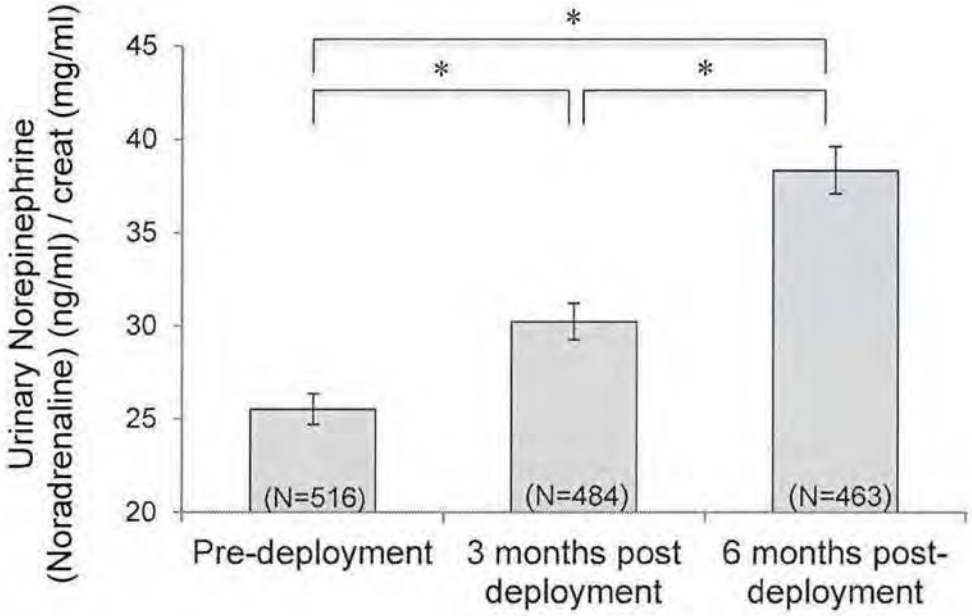
Increase in urinary norepinephrine levels over time



LINEAR MIXED MODEL

OUTCOME: Norepinephrine levels

PREDICTORS	F-value	p
Assessment	54.96	<.0001
Age	0.10	0.749
Prior deployment	0.86	0.354
Cohort	3.97	0.008
BMI	0.02	0.891
Ancestry component 1	5.06	0.025
Ancestry component 2	0.01	0.923
Ancestry component 3	0.06	0.801

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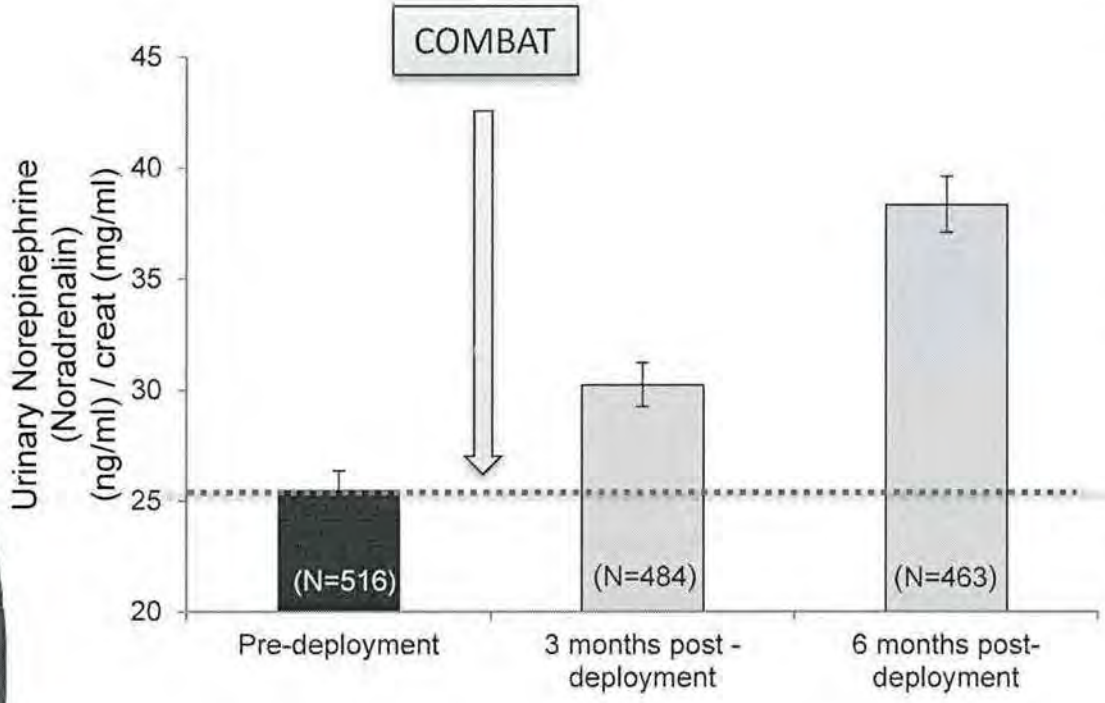
Stress predicts increase in plasma norepinephrine level

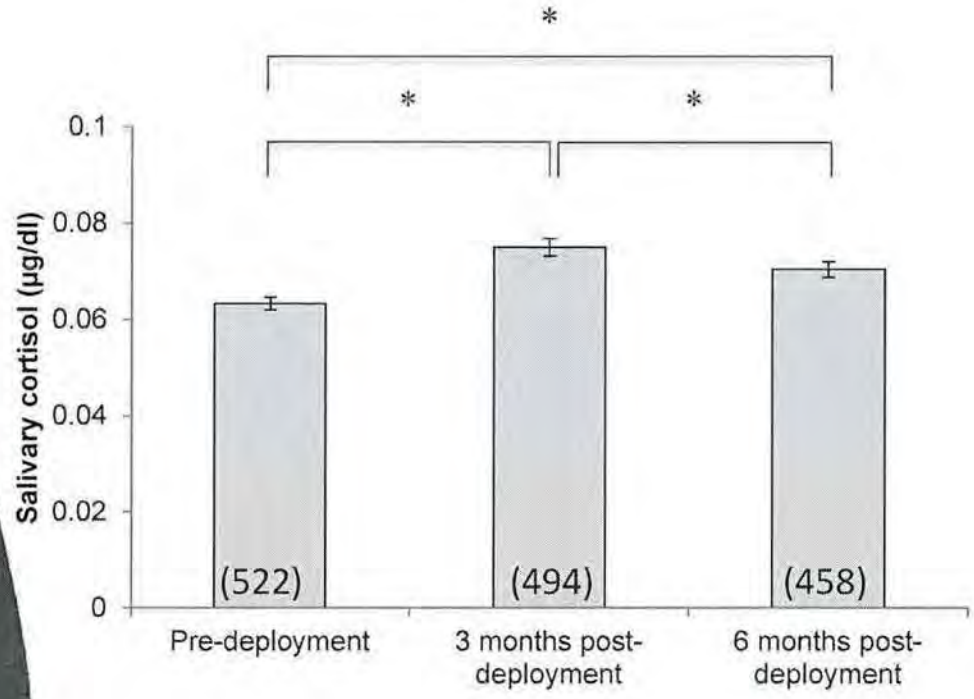


LINEAR MIXED MODEL

OUTCOME: Δ Norepinephrine (pre- to post-deployment)

PREDICTORS	F-value	p
Assessment	32.15	<.0001
Combat stress	15.44	0.000
Age	1.08	0.298
NE (pre-deployment)	0.12	0.730
Cohort	17.94	<.0001
BMI	0.12	0.730
Ancestry component 1	2.60	0.107
Ancestry component 2	1.18	0.279
Ancestry component 3	0.75	0.387

Changes in salivary cortisol levels over time

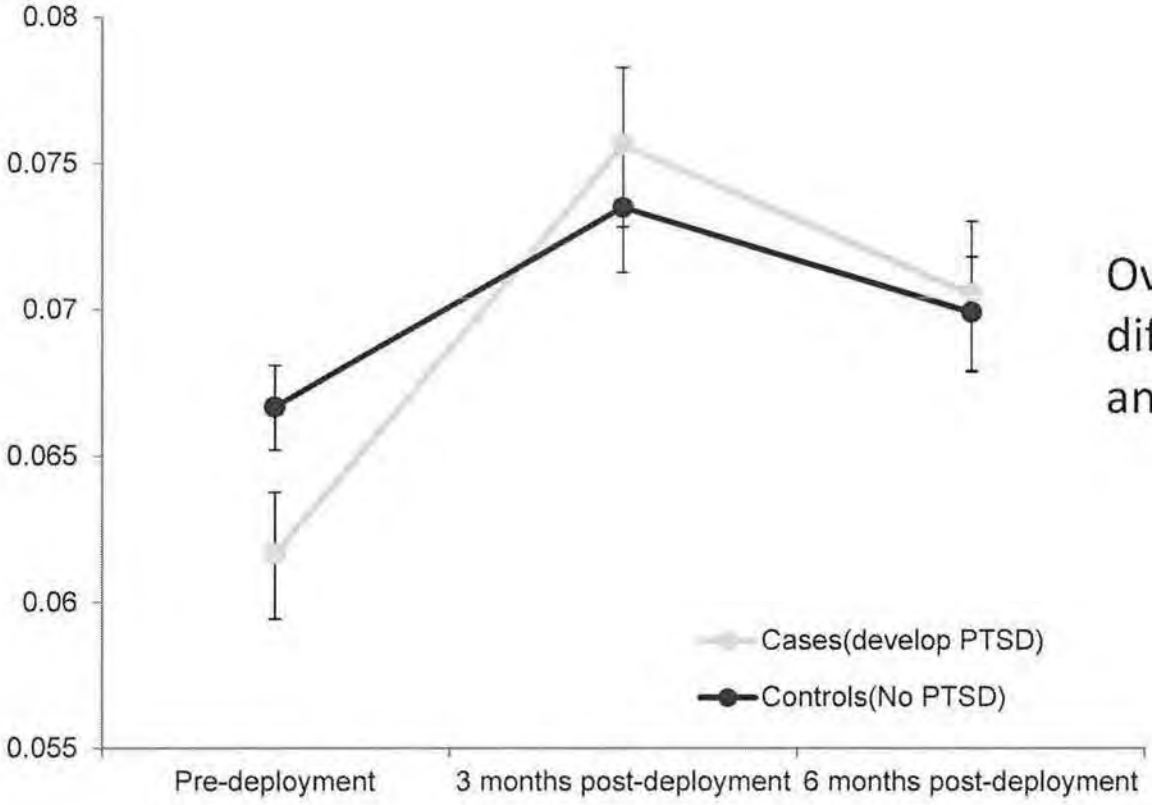


LINEAR MIXED MODEL

OUTCOME: Cortisol levels

PREDICTORS	F-value	p
Assessment	24.38	<.0001
Age	1.62	0.203
Cohort	4.04	0.007
Tobacco consumption	1.37	0.242
Alcohol consumption	0.20	0.652
Time of day	90.19	<.0001
Ancestry component 1	0.13	0.717
Ancestry component 2	0.41	0.520
Ancestry component 3	1.58	0.209

Salivary cortisol changes over time in PTSD cases and controls



Overall no significant difference between cases and controls

N	controls	cases
Pre-deployment	346	176
3 months post-deployment	338	156
6 months post-deployment	317	141

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Acknowledgements

Marine Resiliency Study

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- Richard Hauger M.D., Ph.D., UCSD/CESAMH
- Sami Ali Ph.D., UCSD
- Mingxiong Huang Ph.D., UCSD/VA
- Jennifer Vasterling, Ph.D., Co-I, Boston VA/BU
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- MRS-II Research Team

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- Marine Corps
- Navy Bureau of Medicine
- VA Health Services Research and Development

MRS-II Funding

- Marine Corps
- Administered through Navy Bureau of Medicine

Data Collection Assistance

- CESAMH
- NC COSC, San Diego

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Acknowledgements

Special Thanks to the Marine and Sailor Volunteers

of the

MRS and MRS-II

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Is Deficient Sensorimotor Gating a Pre-Existing Factor in Those That Develop PTSD After Combat Deployment?

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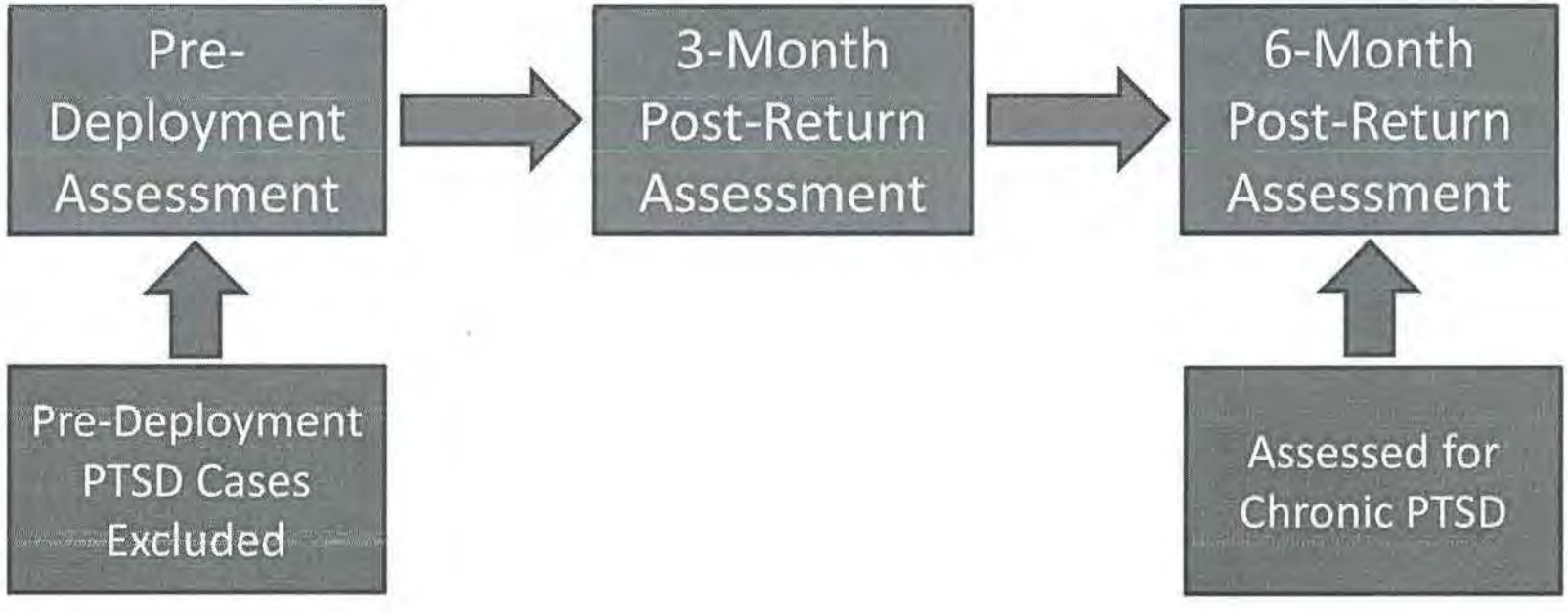
Department of Psychiatry
University of California San Diego
Veterans Affairs Research Service, La Jolla CA

PTSD is a Major Public Health Concern

- > 2.4 Million troops deployed to OIF/OEF
- ~8% PTSD prevalence (Hermann et al., 2012)
- Identifying prospective risk/resilience factors critical for prevention and treatment efforts

Marine Resiliency Study (MRS)

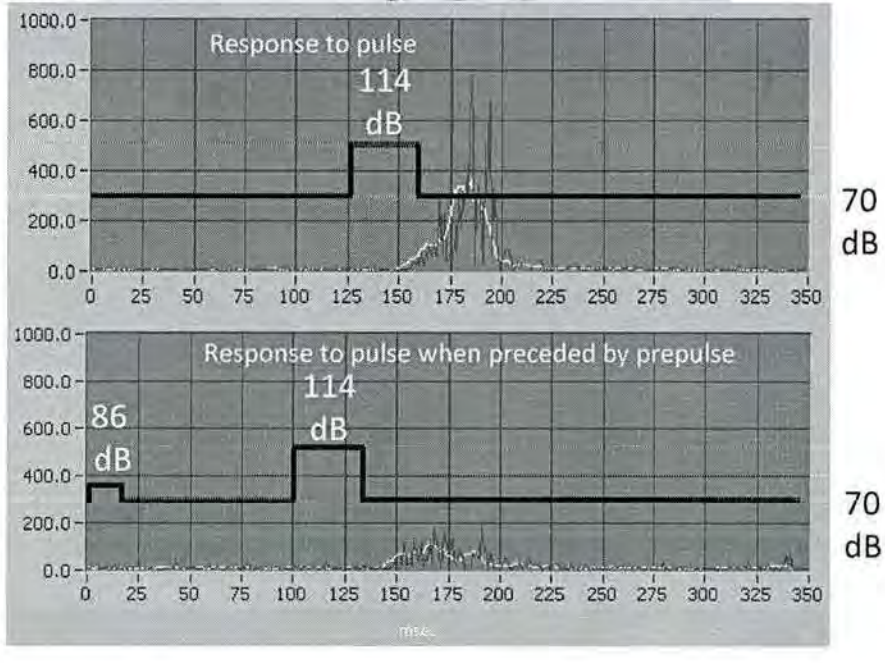


What factors important in who does/does not develop PTSD post-combat?

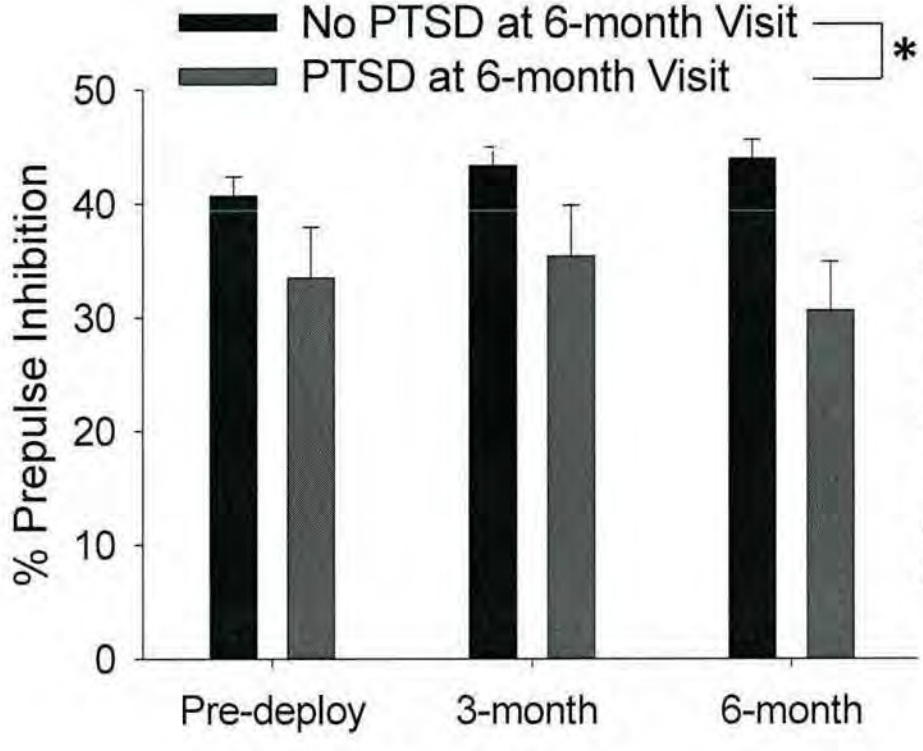
Sensorimotor Gating: Acoustic Prepulse Inhibition

- Stable, heritable measure of "pre-concious" filtering processes
- Brainstem circuit modulated by cortical and subcortical regions
- Deficient across number of psychiatric disorders
  - Unknown role in PTSD

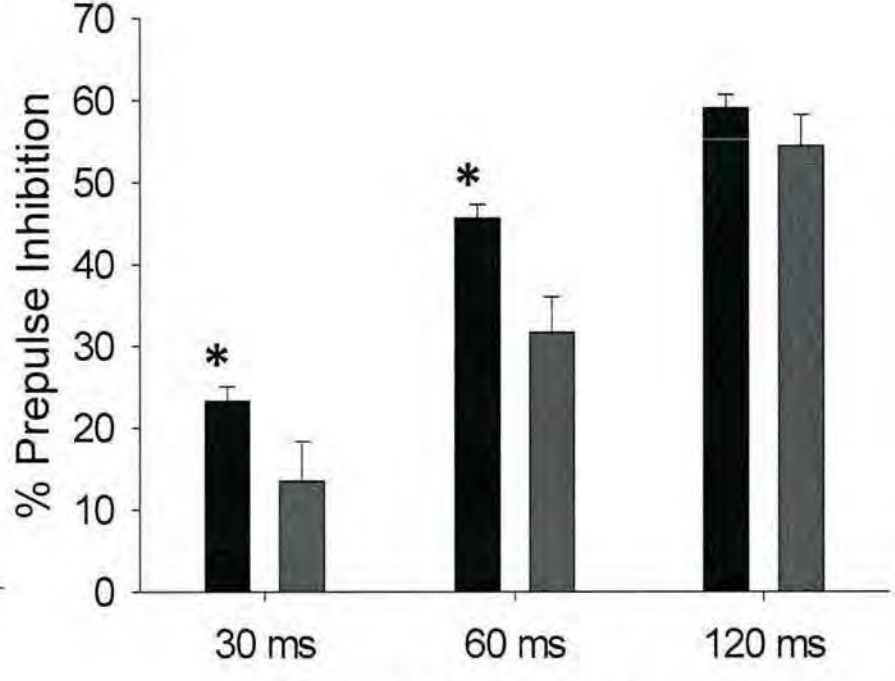
- *Hypothesis:* PPI performance is prospective factor for PTSD development



Participants with PTSD *After Deployment* have ↓PPI both pre *and* post deployment



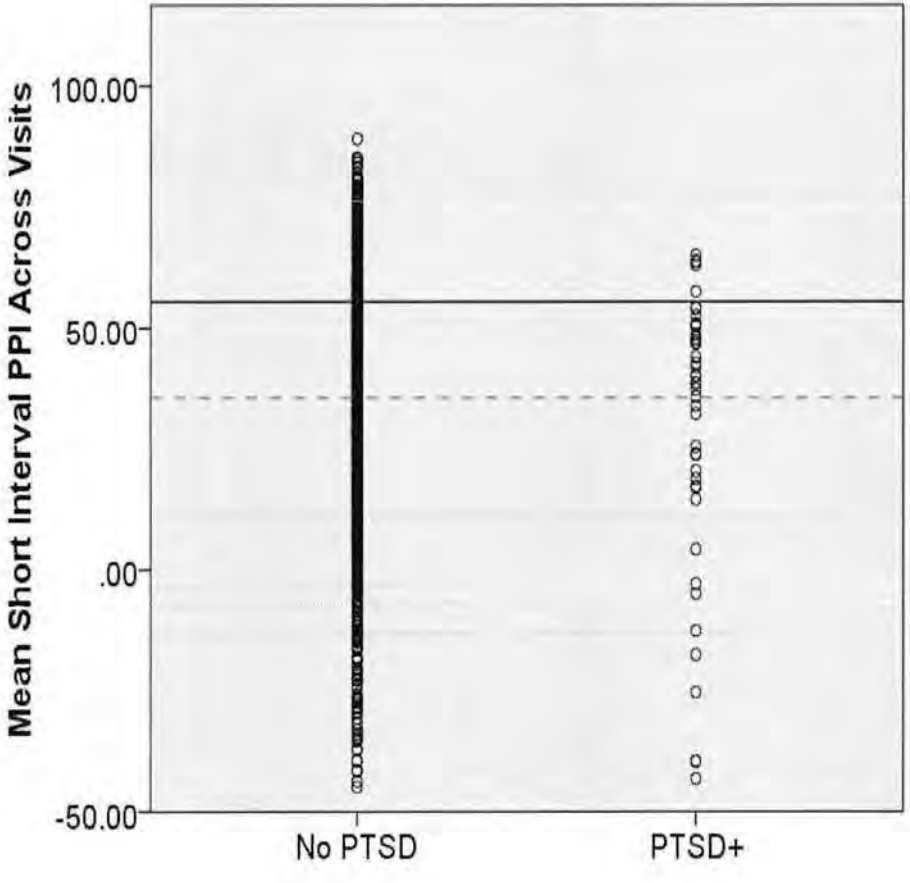
PTSD Main Effect ($p < .005$)

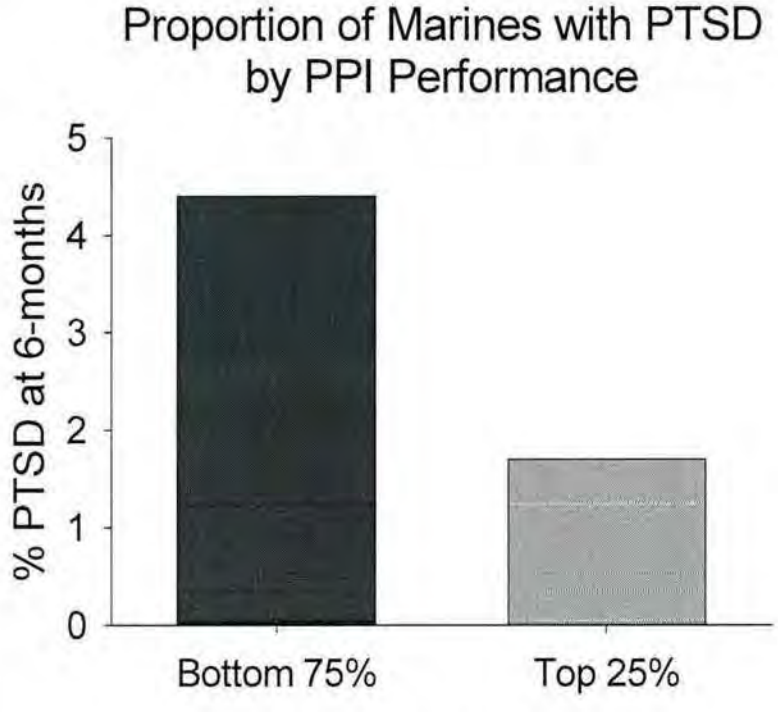


PTSD x isi Effect ($p < .03$)

No PTSD = 1181; New PTSD at 6 month = 46

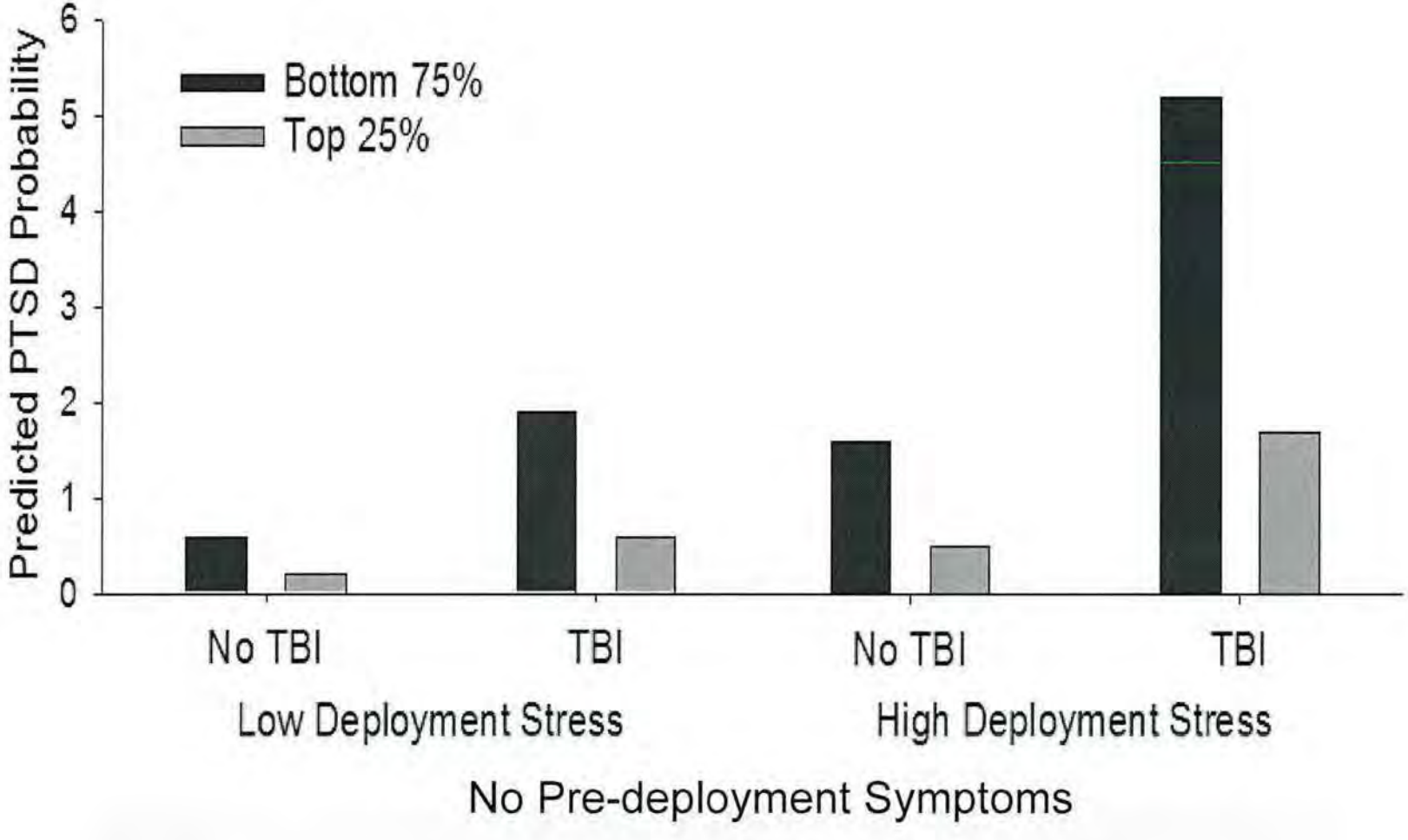
High "Trait" PPI Performance Reduces Probability of Chronic PTSD





$X^2 = 4.3$, $p < .04$

High "Trait" PPI Performance Reduces Probability of Chronic PTSD



Summary/Implications

- PPI appears related to likelihood of chronic PTSD following combat
- Instead of deficient PPI predicting risk, high PPI performance appears to confer resiliency
  - ~ 2/3 ↓ in predicted probability
- Future Directions - May be useful endophenotype for identification of circuits and pathways conferring stress resilience

Acknowledgments

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Poster Time:
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UCSD

DEPARTMENT OF THE NAVY · BUREAU OF MEDICINE AND SURGERY

VA Center of Excellence
CESAMH
Stress and Mental Health

Prospective Examination of Prepulse Inhibition in OIF/OEF Marines Suggests Reduced Sensorimotor Gating is a Pre-Existing Factor in Those That Develop PTSD After Combat Deployment

V.B. Risbrough*[1,2], D.T. Acheson*[1], D. Baker[1,2], C. Nievergelt[1,2], K. Yurgil[2], M. Geyer[1,2]
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Background

- PTSD is a major health concern arising from the wars in Iraq and Afghanistan
- Treatment/prevention efforts require an understanding of the underlying neurobiological processes involved
- Critical to ID components of PTSD-related phenotypes that are pre-existing factors

Prepulse Inhibition (PPI)

- Measure of sensorimotor gating and pre-attentional information processing (Geyer & Braff, 1987)
- Acoustic "prepulse" 30-300 ms before a startling stimulus reduces startle magnitude, possibly via direction of attentional resources toward the prepulse creating a "gate" (Swerdlow et al., 1999)
- Well-defined neural circuit modulated by both subcortical and cortical regions
- Deficient in a number of disorders, though role in PTSD currently unclear
- Hypothesis: Deficient sensorimotor gating (i.e., PPI) is a pre-existing factor in development of PTSD

Methods

Data collected as part of the Marine Resiliency Study (MRS), a prospective study of Marines deploying to Iraq of Afghanistan

Assessment Visits

1. Pre-Deployment
2. 3 months after return
3. 6 months after return

Measures

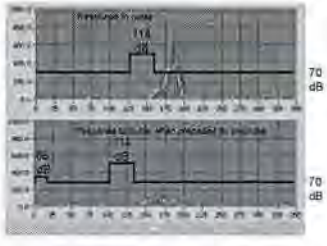
PPI task (See Acheson et al., 2012)
Interstimulus intervals (isi): 30, 60 & 120 ms

Clinician Administered PTSD Scale (CAPS)
Establish PTSD diagnosis

Deployment Risk and Resilience Inventory-2 (DRRI-2)
Assess deployment-related stress and trauma

Traumatic Brain Injury (TBI) Interview
Deployment TBI (defined by LOS or AMS)

PPI Testing



Sample and Exclusions

1,496 Marines and Navy Corpsmen from 4 separate Infantry Battalions

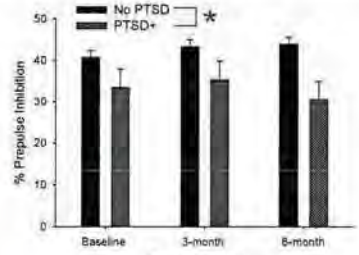
- 143 (9.5%) excluded: poor startle response/technical problems
- 126 (8.4%) excluded: met PTSD diagnostic criteria pre-deployment

PTSD Incidence by PPI Performance

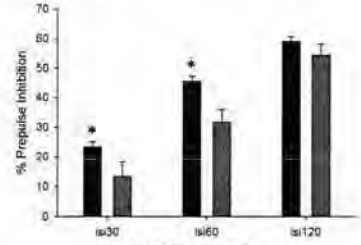
PPI Performance	With PTSD	Without PTSD
Top 25%	5 (1.7%)	286
Bottom 75%	41 (4.4%)	895
Total Sample	46 (4%)	1,181

$X^2 = 4.3$, $p < .04$

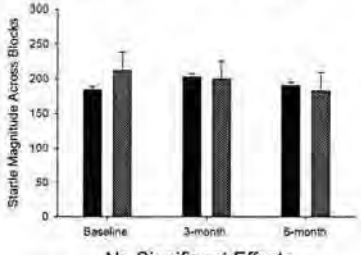
Mixed Model Analysis of PPI and Baseline Startle



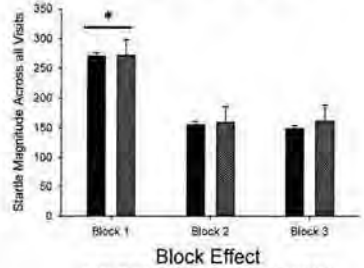
PTSD Effect
$F(1,1348)=7.74$, $p < .005$



PTSD x isi Effect
$F(2,5608)=3.56$, $p < .03$



No Significant Effects



Block Effect
$F(2,7841)=264.82$, $p < .0001$

*** = $p < .05$**

Logistic Regression Analysis

Predictor	β	Wald	p	Odds Ratio	95% CI (UL – LL)	
Pre-Deployment CAPS Symptom Severity	.04	12.8	<.001	1.04	1.02	1.06
Deployment Stress (DRRI-2)	.53	6.65	<.01	1.7	1.61	3.39
Deployment TBI	1.24	12.6	<.001	3.44	1.74	6.8
Top 25% PPI Performance	-1.14	5.39	<.02	.32	.123	.837

Data Analysis

PPI

- 100-[100 x (mean prepulse trial/mean startle trial)]

DRRI-2 Composite Score

- Calculated by computing a Z-score for each subscale and averaging across all 4 subscales
  - Combat Experience, Post-Battle Experience, Perceived Threat, Difficult Working/Living Environment

Mixed Effects Models

- PPI and Baseline Startle
  - Fixed Factors: 2 (PTSD) x 3 (Startle Block or isi) x 3 (Visit)
  - Random Factors: Subjects within Battalion

Logistic Regression Factors

- "Trait" PPI: average of isi30 and isi60 across all visits
- Top Quartile defined as "High Performers"
- Also pre-deployment symptoms, deployment stress, and TBI
- Battalion did not significantly improve model

Main Results and Summary

Mixed Effects Models

- PPI in No PTSD > PTSD+ across all visits and isi
- Overall group difference at both 30 and 60 ms isi
- No effect of, or interactions with, Visit
- No effect of PTSD on baseline startle or habituation

Logistic Regression

- Top quartile PPI performance appears to confer resiliency , reducing predicted probabilities by ~2/3 across all levels of the other predictors

Summary

- PPI appears to be related to the likelihood of developing PTSD following combat
- High PPI performance may represent a pre-existing resilience factor in this sample
- PPI may be a useful endophenotype for further investigation of biological mediators of resiliency to stress injuries

Funding

Navy BUMED, VA HSR&D, Marine Corp, NHRC

Brief
MRS-II Update

1st MARDIV
HQMC
VA
NIMH
Navy
UCSD

Dewleen G. Baker M.D.

Professor, Department of Psychiatry
University of California, San Diego
Director of Research
VA Center of Excellence for Stress and Mental Health

Context: History and Background MRS-II

•Army Study to Assess Risk and Resilience in Service Members (STARRS)

- Initially established as a suicide prevention study
- Largest study of active duty military ever established; 5 year study (2011-2014)
- Expanded to include overall mental health risk and resilience factors as well as suicide
- Marine Corps was included within Army STARRS, but the Marine Corps sought a study specific to Marines and Marine Corps culture
- But the power (number of Marines) is too small to successfully complete an entirely separate study focused on suicide prevention
- The Marine Corps, the National Institutes of Mental Health (NIMH), and Army STARRS jointly decided to leverage the already existing Marine Resiliency Study as a separate, but coordinated study with Army STARRS

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Progress and Development MRS-II

•Coordination of the Marine and Army STARRS study was assigned to Marine Family Behavioral Health Branch, HQMC

• MRS II developed through joint discussions of HQMC, NIMH, VA San Diego and UCSD researchers

• Memorandum of Agreement developed between HQMC with NIMH to oversee the conduct of the study.

• Funding for MRS II through Advanced Medical Programs, Naval Medical Research Center

•Data collection began Sept 2011, is ongoing

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MRS-II: Key Personnel

1st Marine Division
MajGen Lawrence Nicholson

5th Marines
Col Jason Bohm

7th Marines
Col Jay Bargeron

7th ESB
LtCol John Martinko

Marine & Family Programs, M&RA, HQMC
BGen. R.A.C. Sanborn

Behavioral Health Branch
Dr. Keita Franklin
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National Institute of Mental Health
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Army

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RADM Bruce Doll

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  jha9030811@aol.com
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MRS & MRS-II Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews

Historical
- Self-report questionnaires

Neuropsychological
- Attention, Memory, Executive Function, Reasoning, Social Cognition (AMAM & GUR)

Metabolomics
Imaging (DTI/MEG)

MRS
Secure database

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Biological assessments

Biomarkers
- NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- Startle threshold and habituation, fear conditioning and extinction, heart rate variability

Genomics

- **GWAS (complete data)**
- **Methylome (subset, pre-post)**
- **Transcriptome (subset, pre-post)**

MRSII Brief Topics

- Contribution of deployment-related TBI event and post-deployment PTSD
- Diffusion Tensor Imaging (DTI) and Magnetoencephalogram (MEG) findings and plans
- Other analyses in process
  - Blood biomarkers – early findings, metabolomics

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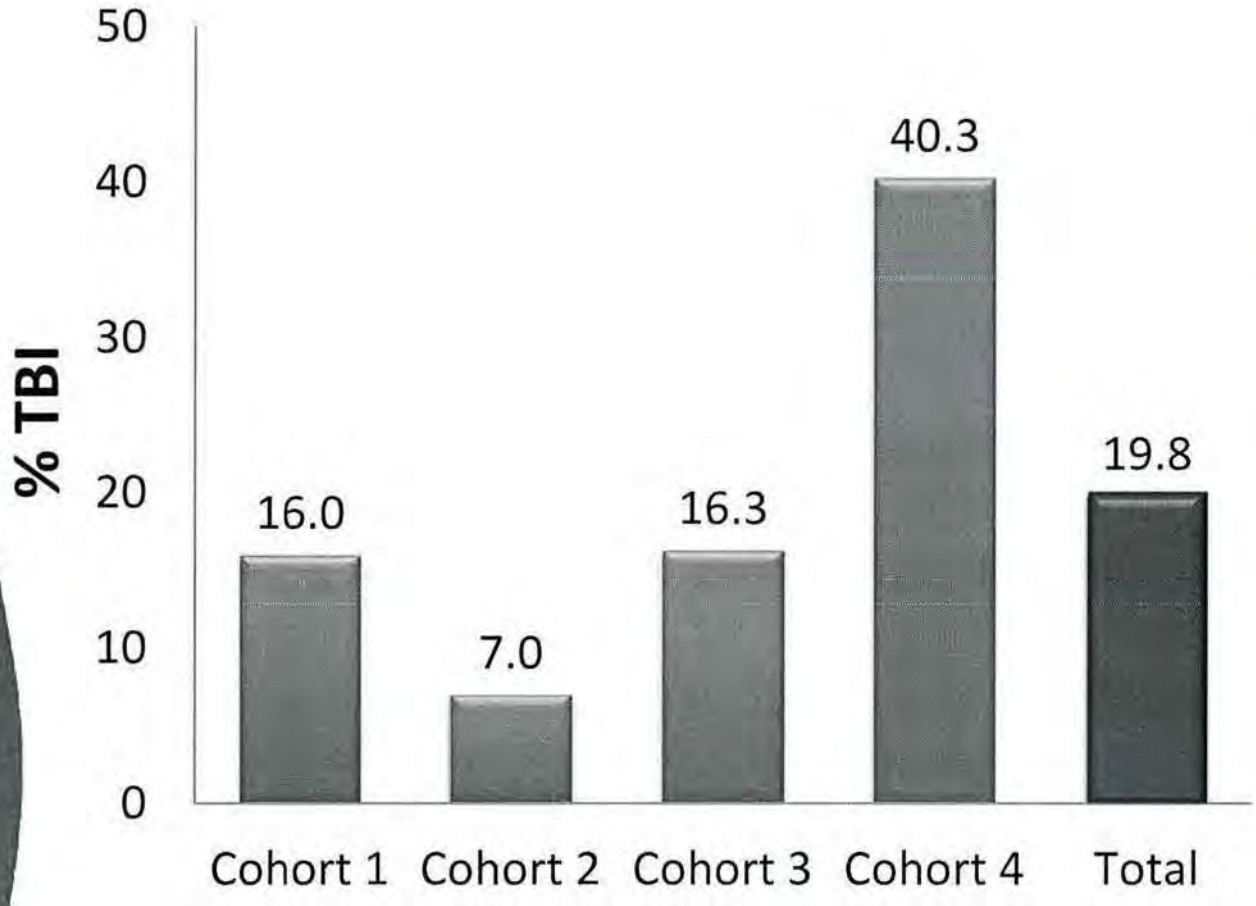
Deployment-related TBI endorsement was variable across deployments, but was high in some battalions



Combat Experience Scale	
Cohort 1	6.5
Cohort 2	6.9
Cohort 3	13.2
Cohort 4	22.7
Total	**12.9**

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Marine Resiliency Study Findings

Association Between Traumatic Brain Injury and Risk of Posttraumatic Stress Disorder in Active-Duty Marines

Yurgil KA, Barkauskas DA, Vasterling JJ, Nievergelt CM, Larson GE, Schork NJ, Litz, BT, Nash WP, Baker DG for Marine Resiliency Study Team

JAMA Psychiatry 2014 71(2):149-57

- At the pre-deployment assessment, 56.8% of participants reported prior TBI & At the post-deployment assessment, 19.8% reported a deployment-related TBI

- Deployment-related TBI nearly doubled risk for post-deployment PTSD

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Rationale for MEG study

- War-related TBI is not new. TBI became prevalent during WWI/II and is a signature injury in the OEF/OIF/OND conflicts

- Self-reported TBI and PTSD symptoms
  - Are invisible injuries
  - Show considerable overlap
  - Diagnoses are hard to disentangle clinically

- Medicine is developing better tools to:
  - Visualize PTSD & mTBI injuries and discriminate between them

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MEG Study

MRS-II MEG Study

- Funding: VA Merit Review Grants (PI: Huang, Lee, Canive), Marine Resilience Study-II (MRSII PI: Baker, Exploratory MEG Project PI: Huang; Co-I: Victoria Risbrough), NFL (PIs: Huang, Lee), McDonnel Foundation via Brain Trauma Foundation (PI: Ghajar; site PIs: Lee, Huang)
- Investigator Collaboration: VA San Diego Healthcare System, and University of California San Diego.

Mingxiong Huang
Victoria Risbrough
Dewleen Baker
Annemarie Angeles
Ashley Robb

Roland Lee
Sharon Nichols
Mithun Diwakar
Tao Song

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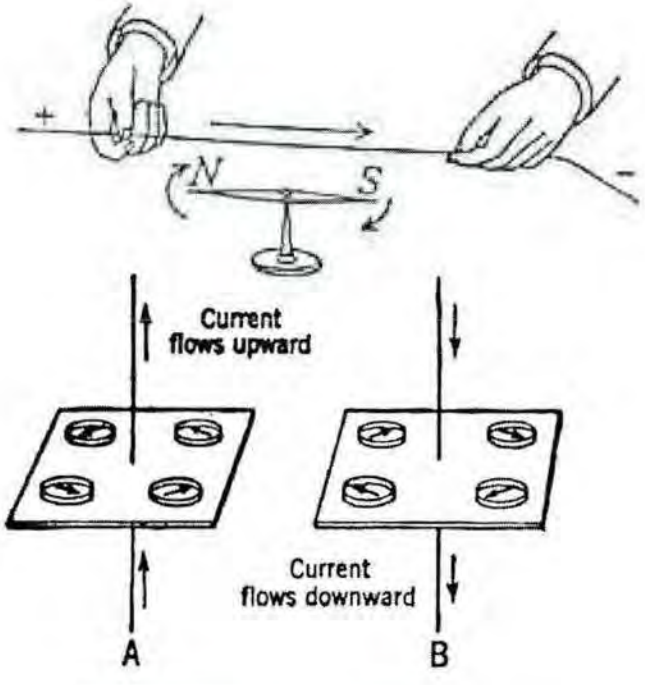
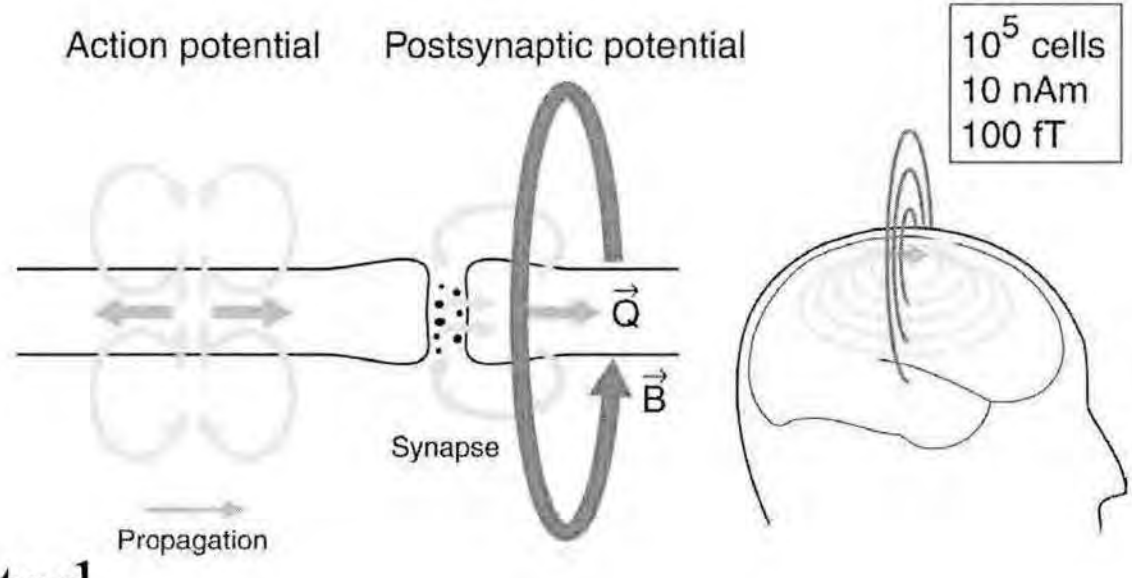
Electromagnetism



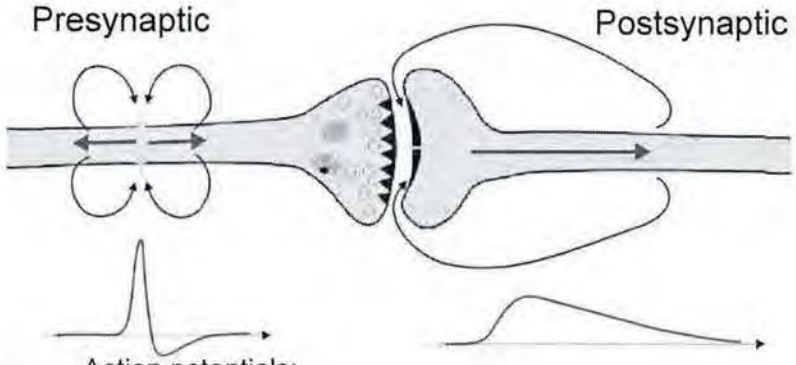
H.C. Ørsted, 1820

Electric current always generates magnetic fields



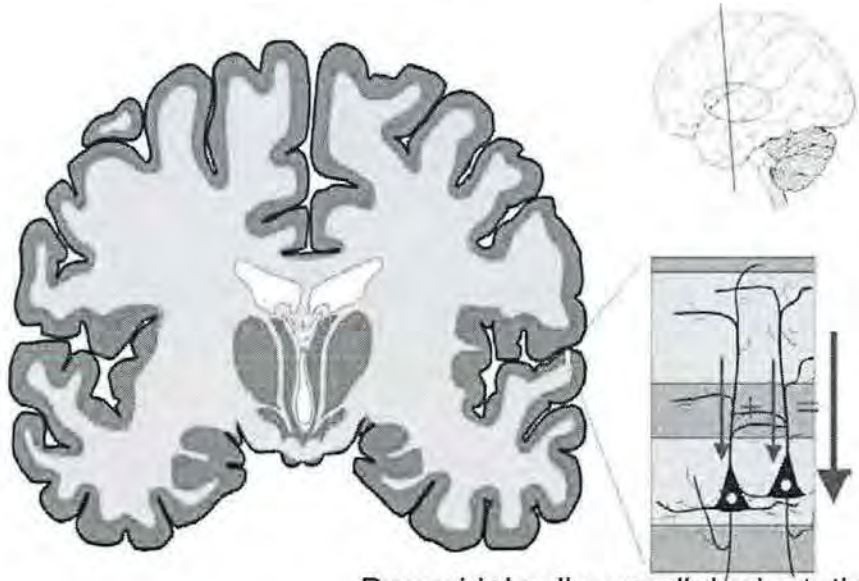
The generation of MEG signal from neuronal current in gray-matter neurons in cortex

Neuronal currents in axons and dendrites

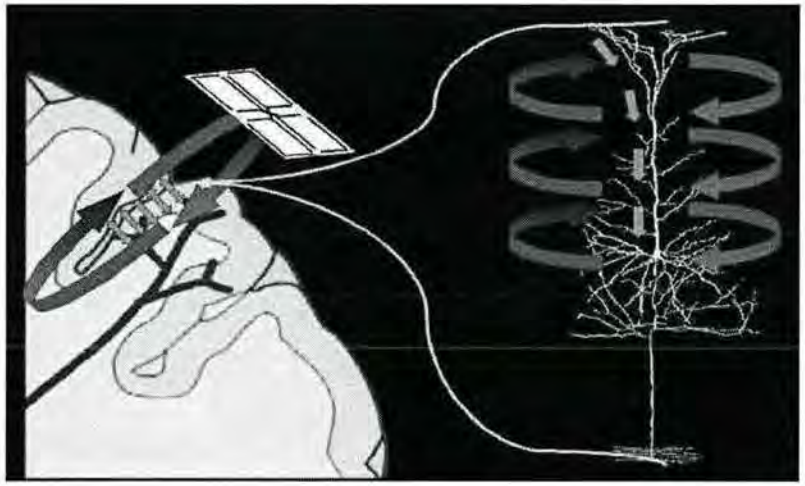


- Action potentials:
  - Fast: no/little temporal summation
  - Cancellation: fields diminish rapidly

Parallel dendrites

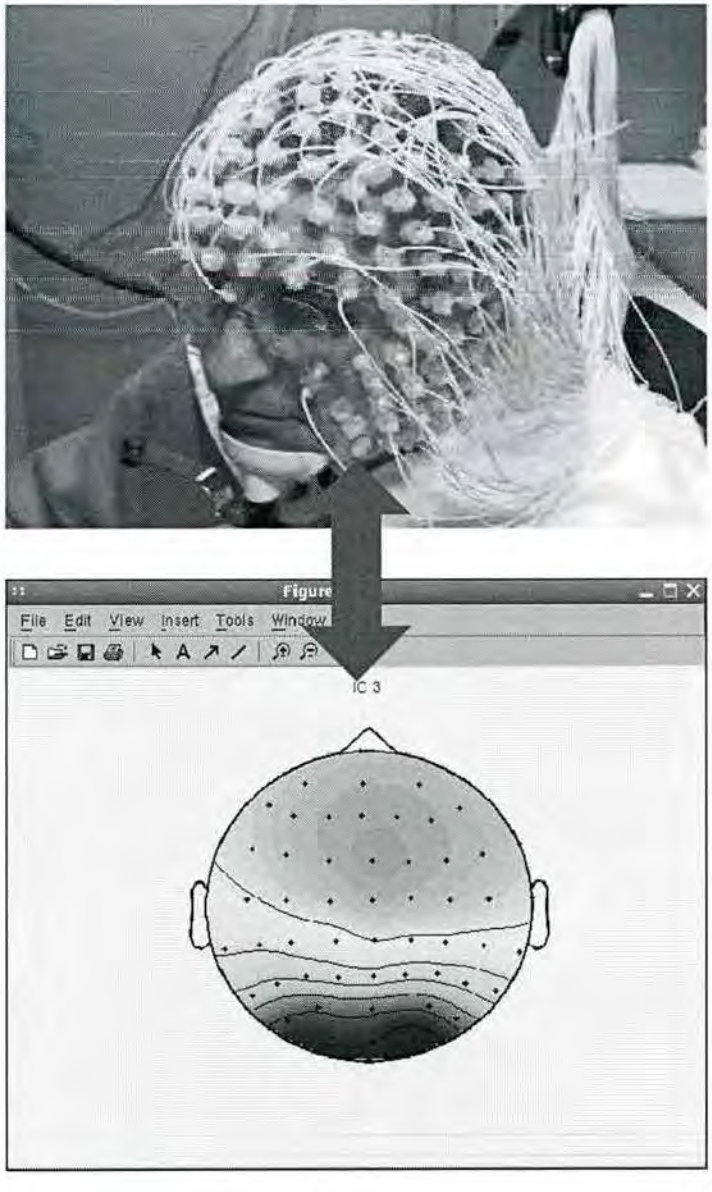


Pyramidal cells: parallel orientation
=> spatial summation



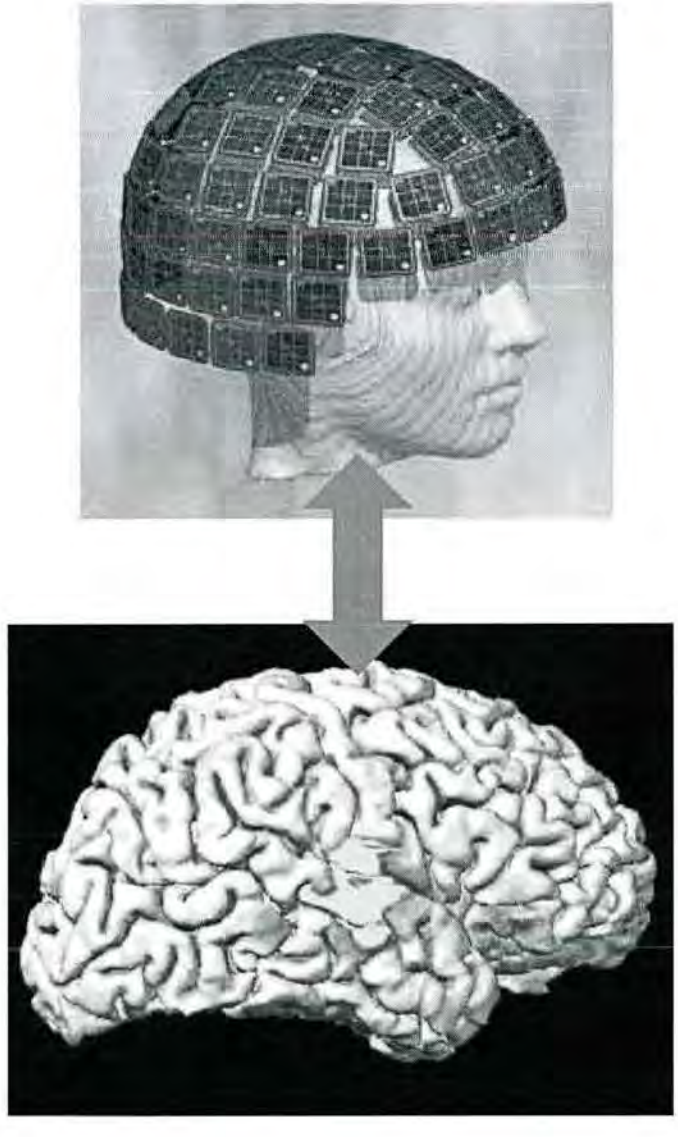
EEG vs. MEG Technology

EEG

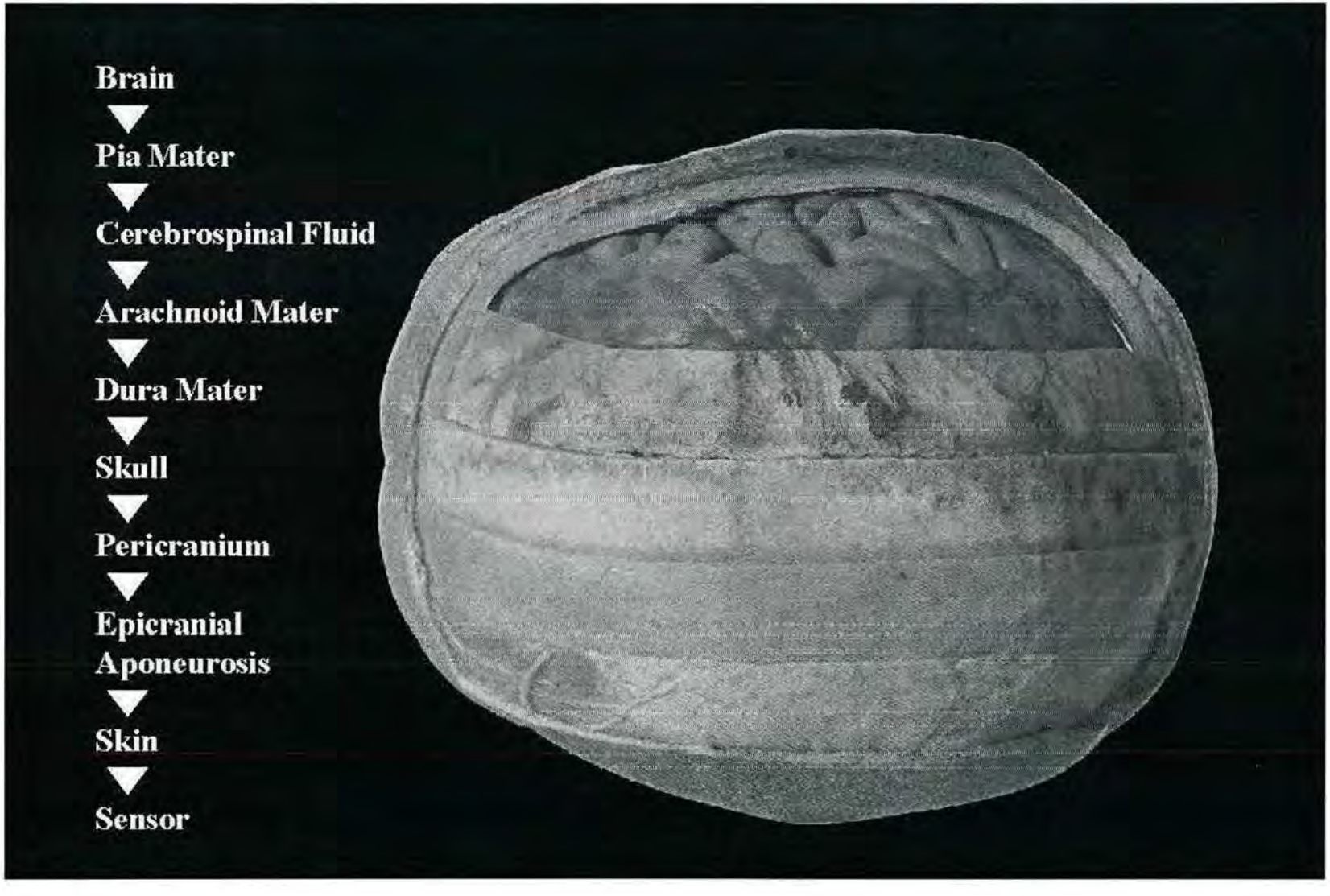


MEG

MEG SQUID Sensor Array

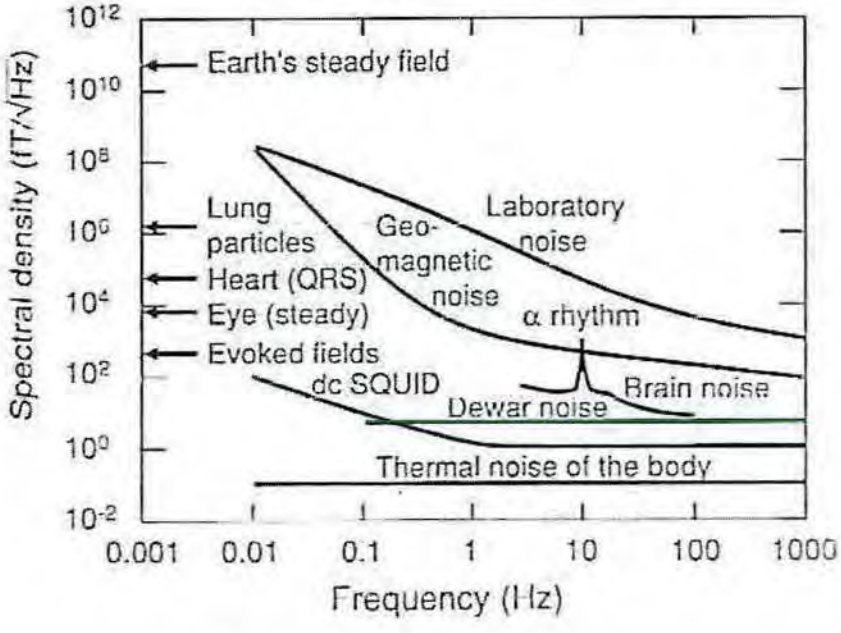


MEG's better spatial localization accuracy (2-3 mm) than high density EEG (in cm) is due to MEG's insensitivity to conductivity profile of the head tissues



Non-invasive MEG Technique with 1 ms Temporal Resolution and several mm Spatial Resolution in Cortex

MEG signal is weak



IMEDCO Multiple Layer Magnetic Shielded Room Installed at UCSD

Shielding factors:

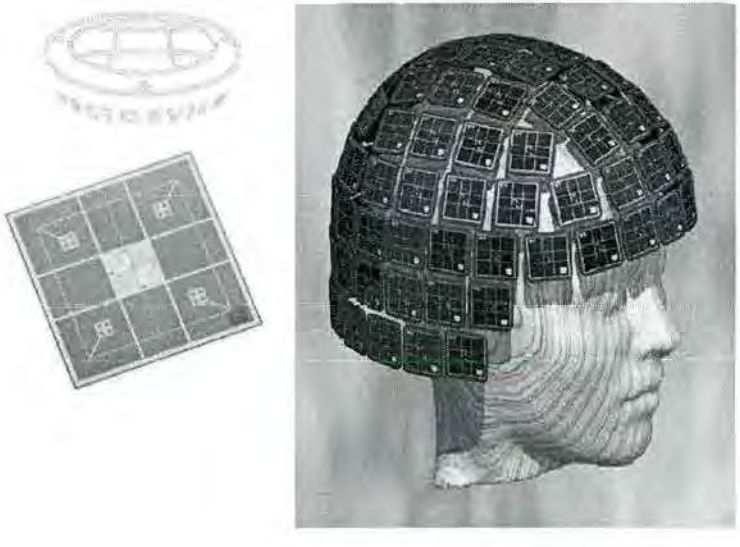
0.01Hz: 65dB

0.1 Hz: 73dB

1 Hz: 108dB

10Hz: 160dB

MEG SQUID Sensor Array



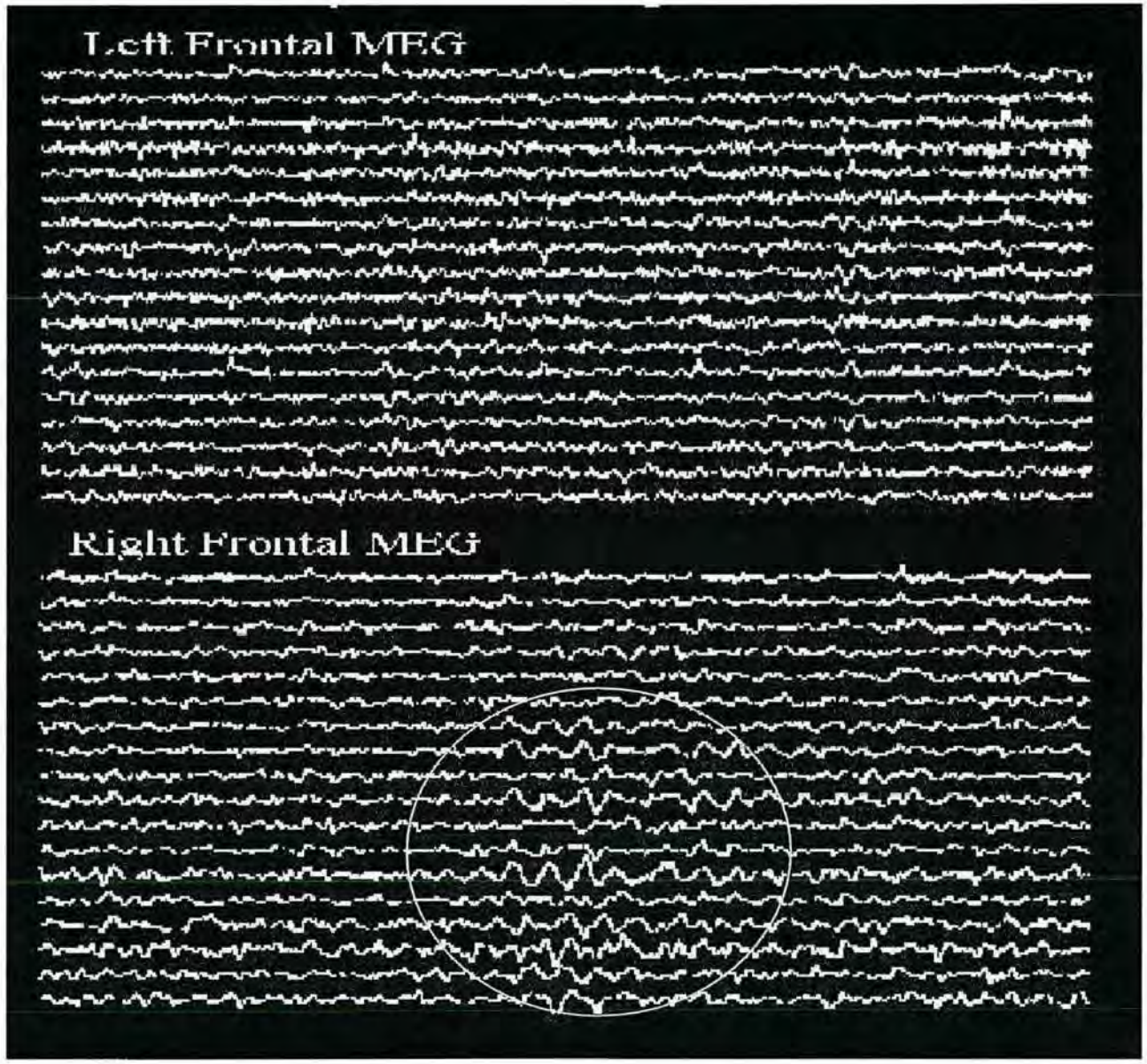
Elekta/Neuromag VectorView Whole Head MEG System with 306 Channels at the UCSD MEG Center

Mild TBI is often referred as an *invisible* injury: Detecting Mild TBI is Challenging using Conventional Neuroimaging Methods

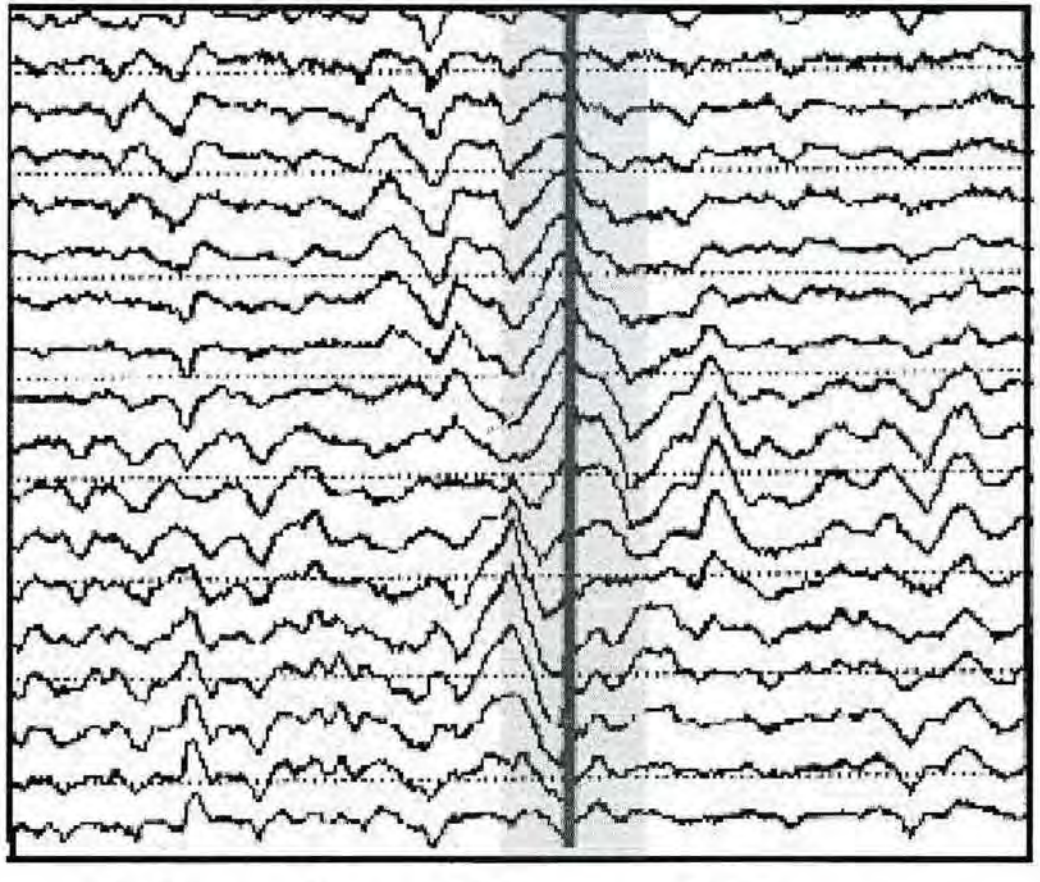
- Traumatic brain injury (TBI) is a leading cause of sustained impairment in veterans, military personnel, and civilian populations.
- Mild TBI (mTBI): injuries are difficult to detect (injuries visible on only 10% of conventional MRIs or CTs).
- Axonal injury is a leading factor in mTBI. Conventional CT and MRI are mainly sensitive to blood product, and less sensitive to axonal damage itself, hence they underestimate the presence of axonal injury, especially in mild TBI cases.
- Injured brain tissues in mTBI patients generate pathological slow-wave magnetic signal that can be measured and localized by MEG (Lewine et al., 1999, 2007).
- Integrate gray-matter MEG slow-wave with white-matter diffusion tensor imaging (DTI) findings in mTBI (Huang et al., 2009)

Abnormal Resting-state MEG Slow-waves in gray-matter (1-4 Hz, delta-waves) are Characteristics of Neurological Injuries in the Brain, resulting from axonal injury

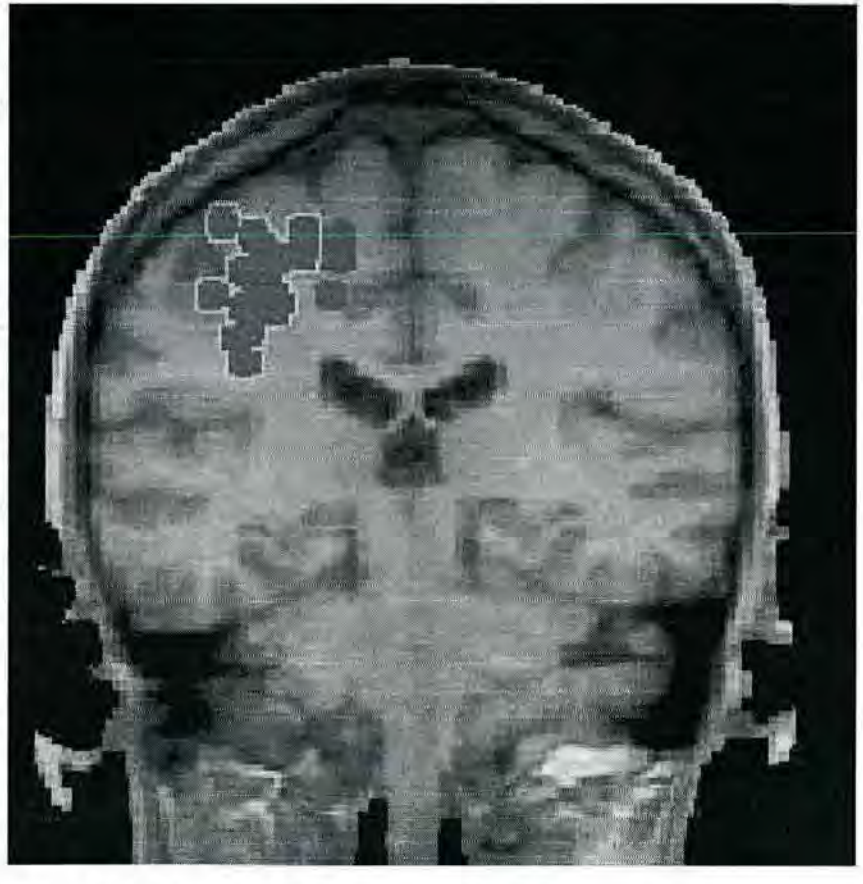
- Stroke
- Brain tumor
- Epilepsy
- Traumatic brain injury



MEG Resting-state Slowing in a mild head trauma patient a with normal MRI



Dipolar Slow Wave



Mild Head Trauma

What is the neurophysiology for resting-state MEG slow-wave generation (1-4 Hz) in TBI?

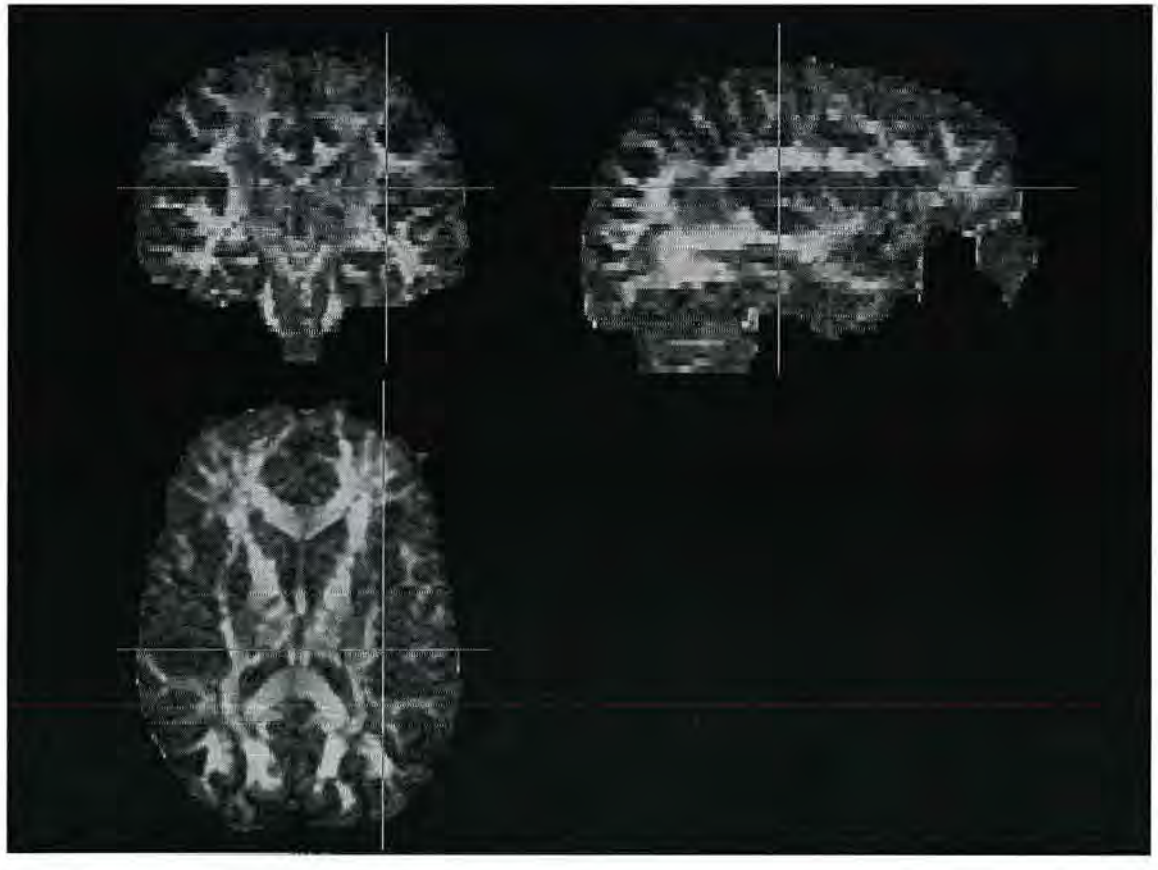
- Animal studies in cats revealed the slow-wave (delta-band 1-4Hz) were due to De-afferentation in gray-matter, caused by axonal lesions in white matter (Gloor et al., Neurology, 1977; Ball et al., Electroencephalogr. Clin. Neurophysiol., 1977).
- Is it possible that abnormal MEG slow-waves in mTBI patients are also due to de-afferentation from axonal injury?
- Our MEG-DTI integration study examines slow-wave in gray-matter and axonal injury in white matter (Huang et al., Journal of Neurotrauma, 2009)

Diffusion Tensor Imaging (DTI)

- DTI is an MR imaging technique based on the Brownian motion of water through tissues
- It measures how easily water molecules move along the direction of white matter fibers versus the directions perpendicular to the fibers.
- TBI causes tissue shearing in the white matter fibers that leads to reduction of DTI signal.

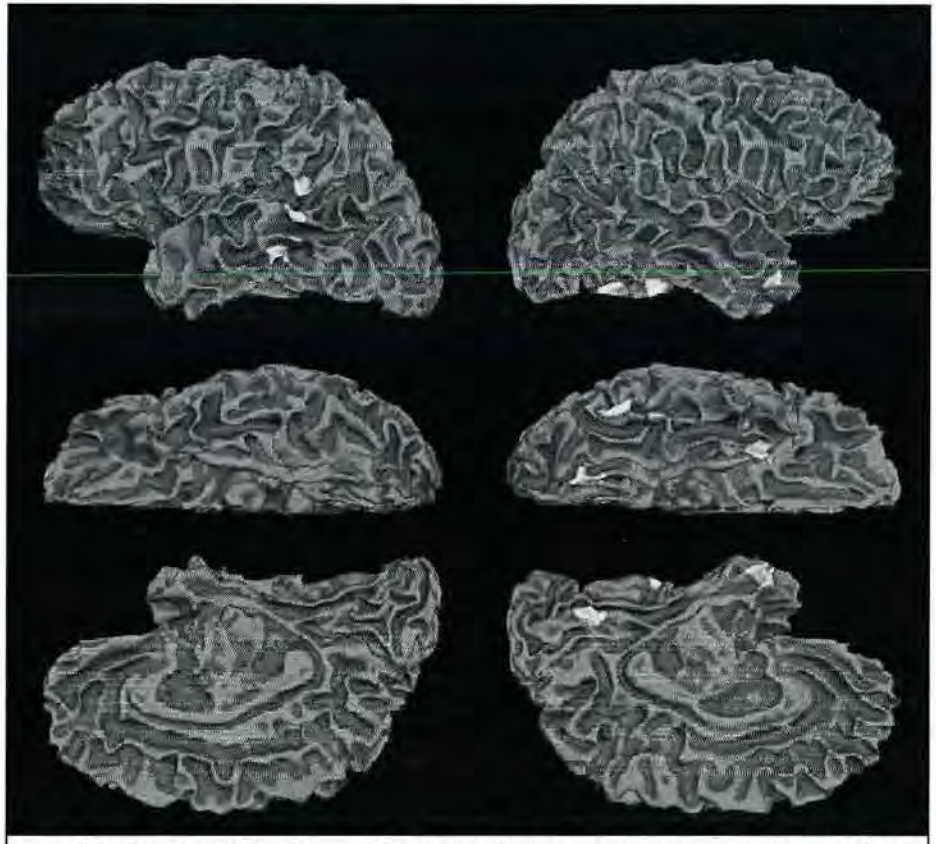
$$D = \begin{pmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{pmatrix}$$

$$FA = \frac{\sqrt{3\left[(\lambda_1 - \bar{\lambda})^2 + (\lambda_2 - \bar{\lambda})^2 + (\lambda_3 - \bar{\lambda})^2\right]}}{\sqrt{2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}}$$

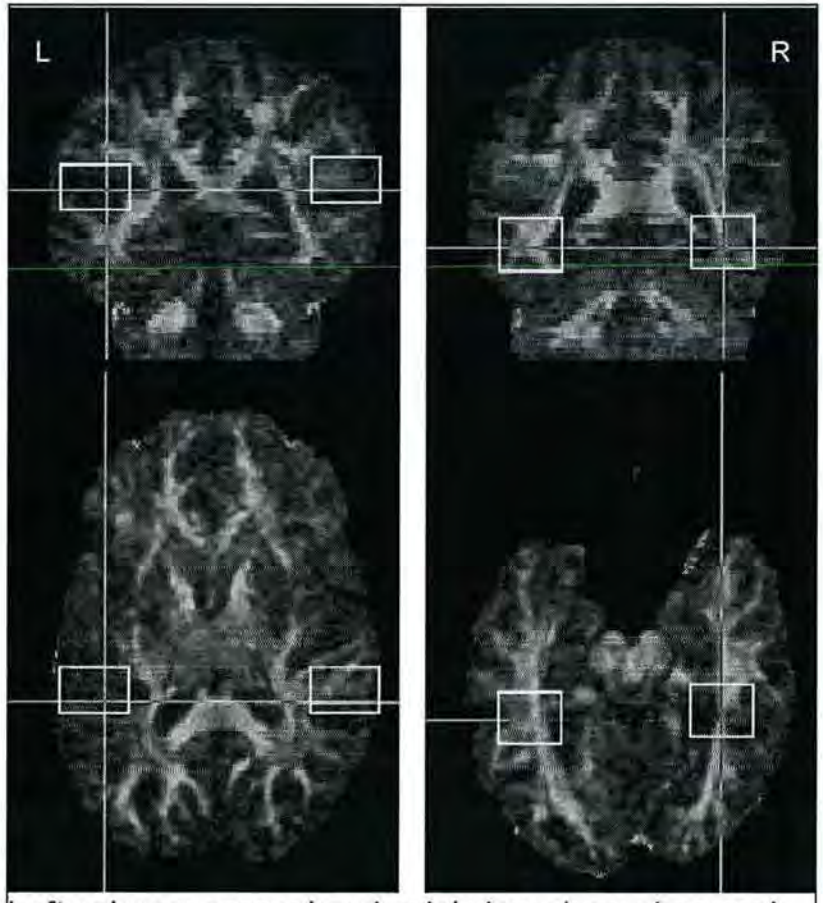


MEG-DTI Findings in Mild TBI due to Sport-related Accidents

History: 17-year old football player, 3 mTBIs. Symptoms: progressive headaches. dizziness, extreme fatigue while performing any mental task, altered sleep, memory problems, changes in speech.
Evaluation: Multiple CT & MRI scans negative.



rs-MEG results show abnormal slow-waves generated from two regions in a TBI patient: 1) left column -- left lateral superior-posterior temporal region, 2) right column --- right inferior-temporal areas. Color threshold $p<0.01$.



Left column: coronal and axial view show abnormal DTI in superior-posterior temporal lobe of the left hemisphere in a TBI patient. Right column: abnormal DTI in inferior-temporal lobe as part of the inferior longitudinal fasciculus of the right hemisphere.

Huang et al., Journal of Neurotrauma, 2009.

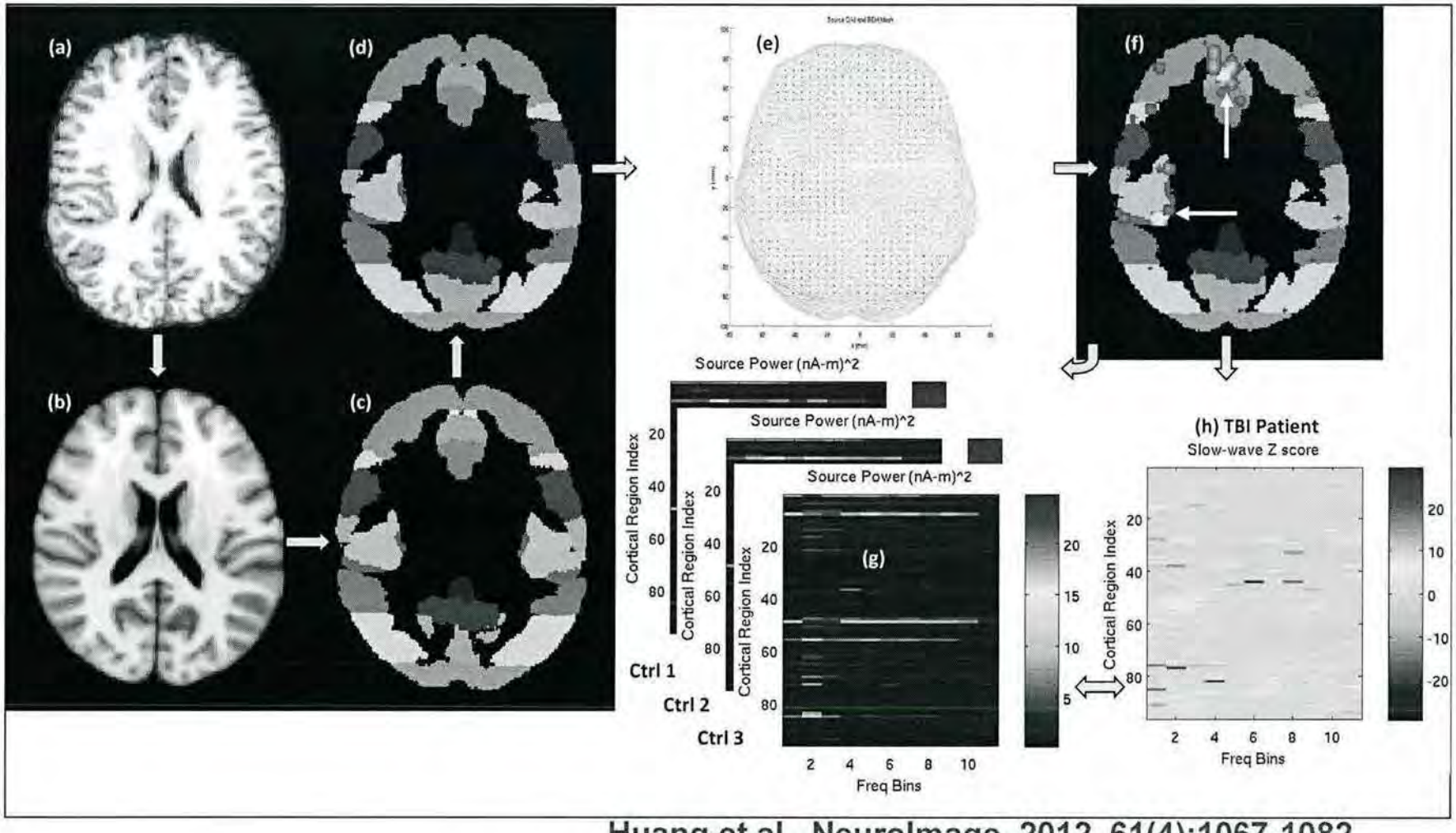
Examining the Positive Detection Rate of Mild TBI using resting-state MEG

- Resting-state MEG data (spontaneous recording with eyes-closed for slow-wave detection) were collected using the Elekta-Neuromag VectorView whole-head MEG system.
- Group 1 contains 23 mild TBI patients whose injuries were caused by blast, all with PCS;
- Group 2 contains the 22 mild TBI were injured with non-blast causes (i.e., motor vehicle accident, sports, and fall), all with PCS
- Group 3 contains 10 moderate TBI that were not blast-related, all with PCS.
- Group 4 contains 44 age-matched healthy control subjects.

Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG Slow-wave Source Imaging

- Resting-state MEG data were analyzed using our new improved frequency-domain VESTAL method to obtain the source images for the low-frequency range (1-4Hz). Normative Database from healthy control subjects were used to detect abnormal slow-wave generation in TBI patients.

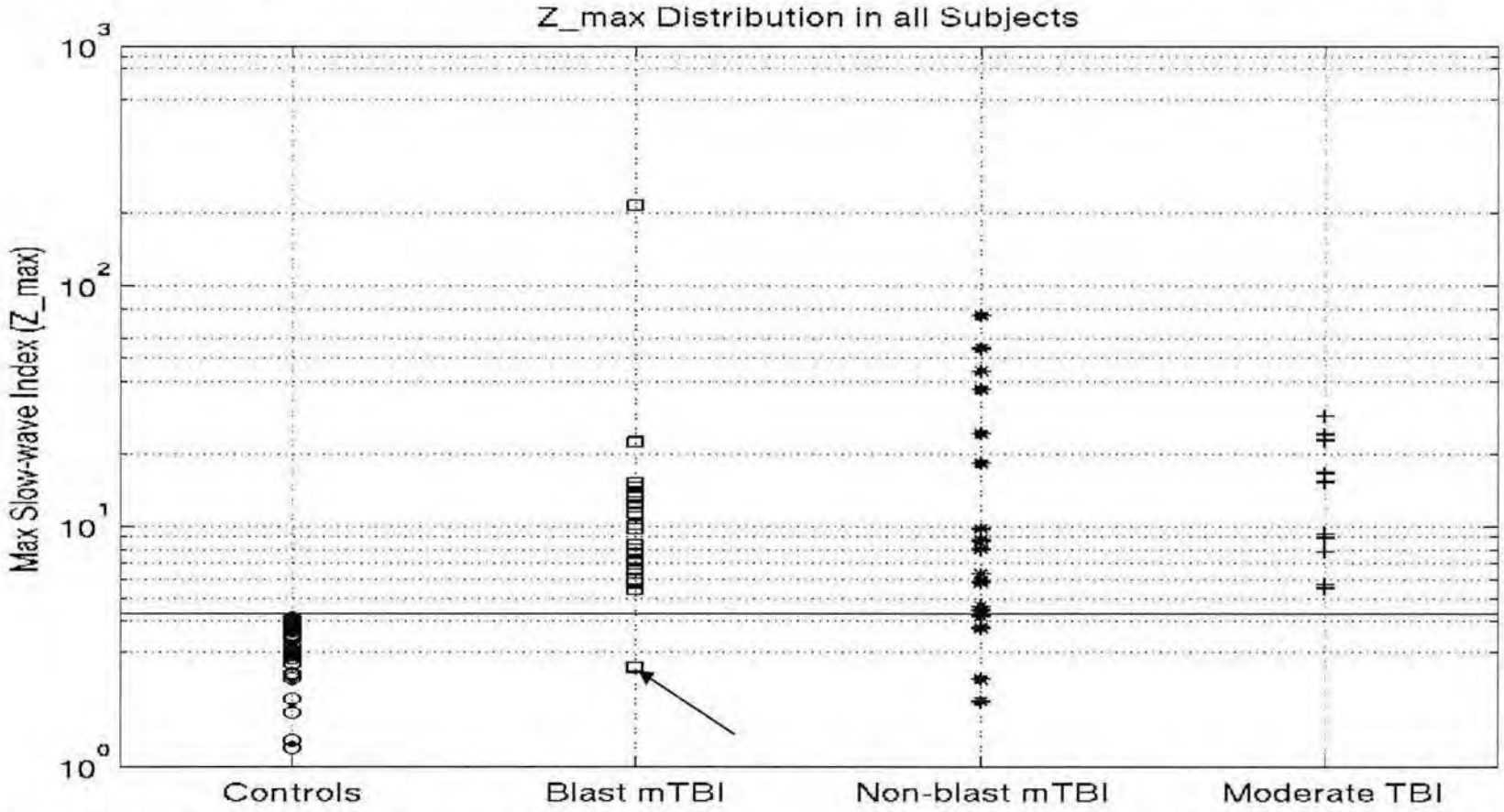


Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG Slow-wave Positive Detection Rate of TBI*

MEG positive-finding rates (**cortical delta wave slowing**) for different TBI groups were calculated at the threshold of 0% false-positive rate in healthy control subjects.

- In the mild TBI group caused by blast, the MEG positive-finding rate: **96%**.
- In the mild TBI group with non-blast causes, the MEG positive-finding rate: **77%**.
- In the moderate TBI group, the MEG positive-finding rate: **100%**.
- In the combined mild TBI group (blast+non-blast), the MEG positive-finding rate: **87%**.



*Being updated with information from MRSII MEG study

Huang et al., NeuroImage, 2012, 61(4):1067-1082.

MEG Slow-wave Exam Correlates with Post-concussive Symptoms

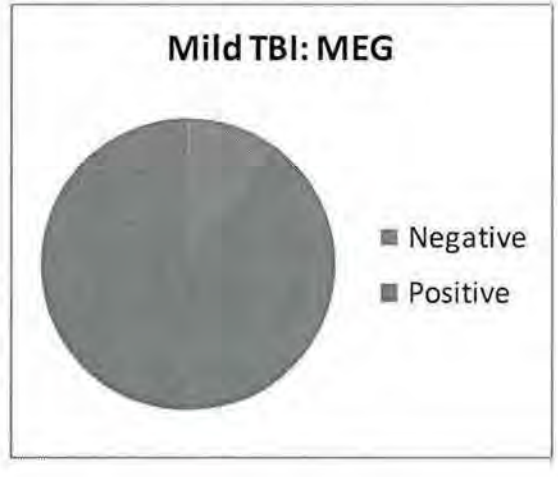
- $N_{slow\text{-}wave\_sum}$ is significantly correlated with N_{PCS_sum} ($r=+0.27$, $p<0.05$) in 55 TBI patients

- Regarding Individual PCS, $N_{slow\text{-}wave\_sum}$ significantly correlated with Personality Changes (e.g., social problems) ($r=+0.32$, $p<0.05$), Apathy, ($r=+0.36$, $p<0.01$), and other visual difficulties ($r=+0.27$, $p<0.05$).

Huang et al., NeuroImage, 2012, 61(4):1067-1082.

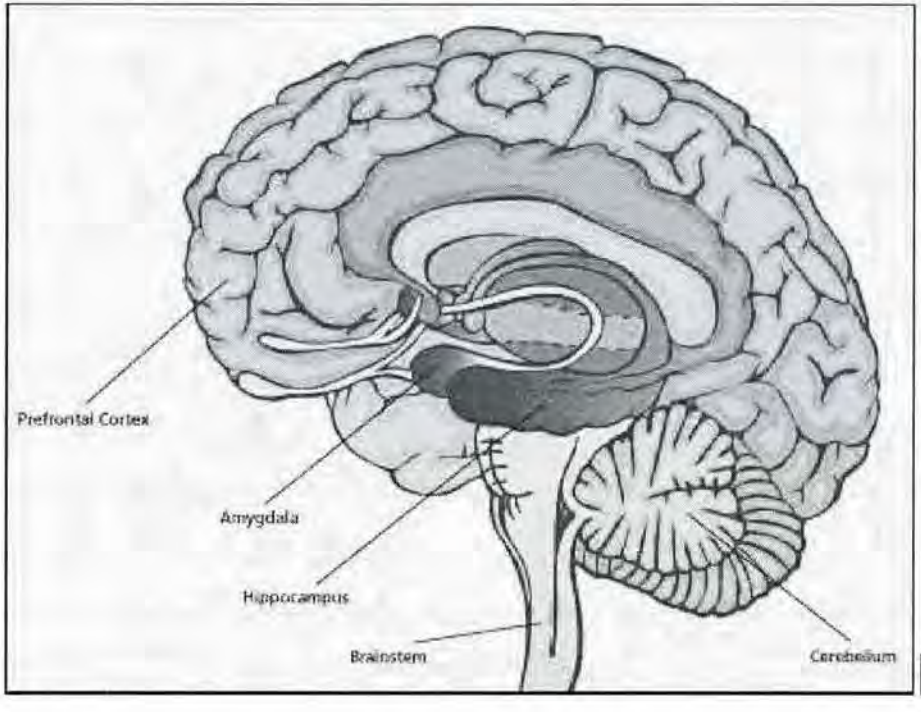
MEG slow-wave imaging marker for TBI*

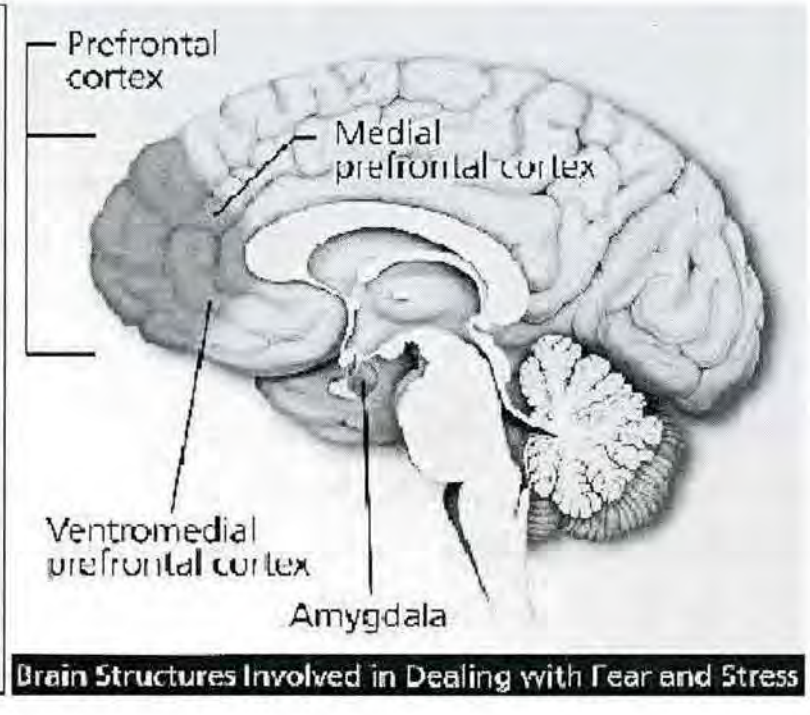
- Positive-detection Rates: MEG slow-waves in TBI patients has **87**% positive-finding rate in mild TBI and **100**% for moderate TBI groups.
- MEG slow-wave findings correlate with post-concussive symptoms
- These detection rates are being updated with a larger normative
- Cohort, and with individuals
- with mTBI



Huang et al., NeuroImage, 2012, 61(4):1067-1082.

"Fear Network" and Inhibitory Function of Ventromedial Frontal Cortex in PTSD





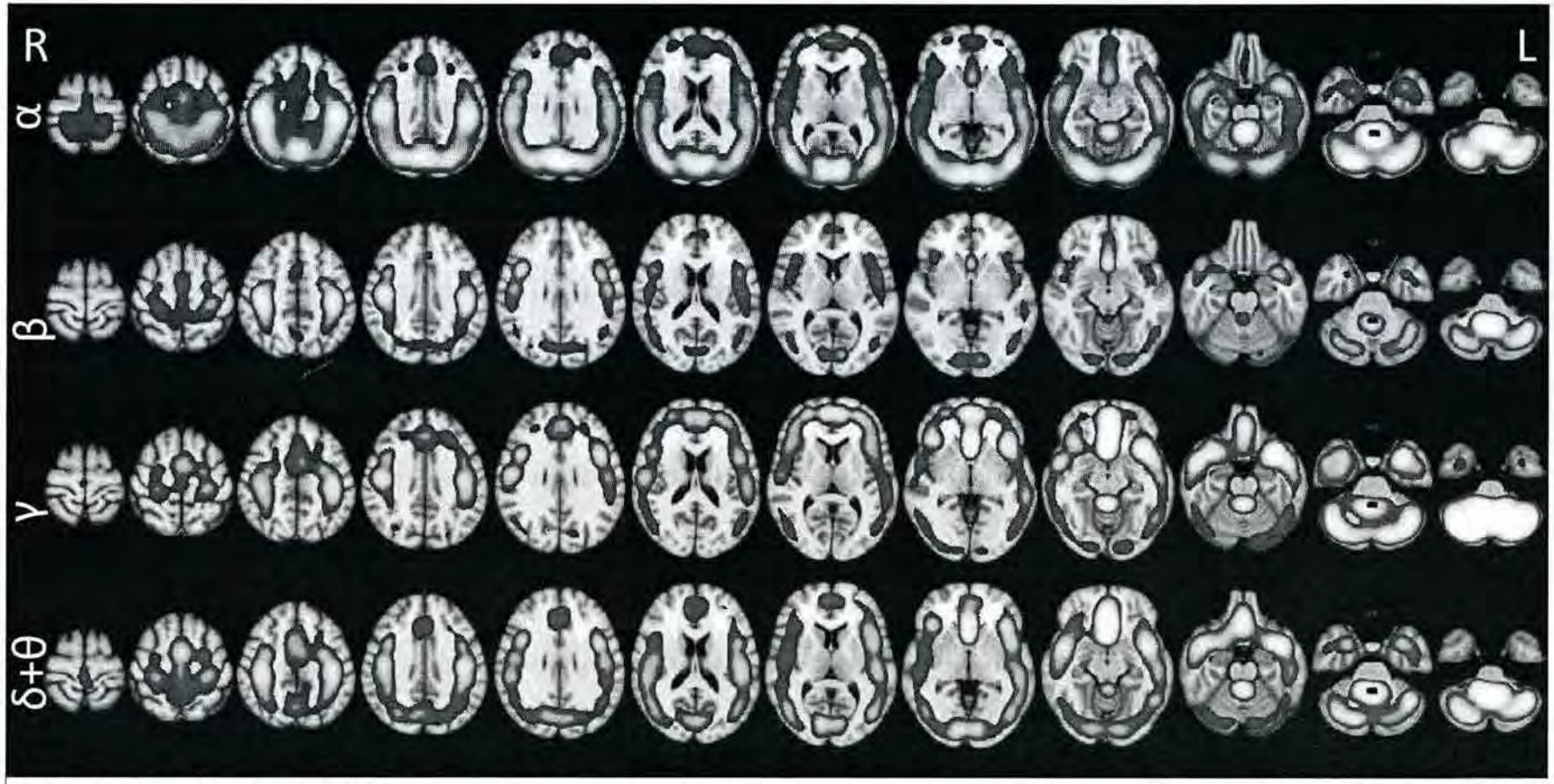
Brain Structures Involved in Dealing with Fear and Stress

- **Hypothesis 1: hyper-activity in "fear network"**
- **Hypothesis 2: hypo-activity in ventromedial frontal cortex, indicating reduced inhibitory signal from frontal cortex to the "fear network"**

Developing MEG source imaging marker for PTSD as well as mTBI: Study I

- Resting-state MEG recording with eyes-closed
- 44 Healthy Controls
- 21 Veterans diagnosed with PTSD (CAPS total: 53-92)
- MEG source imaging for gamma band activity (30-100 Hz)

First Whole-head Source Amplitude Images of Brain Rhythms for Different Frequency Bands since German Physiologist Hans Berger in 1924?

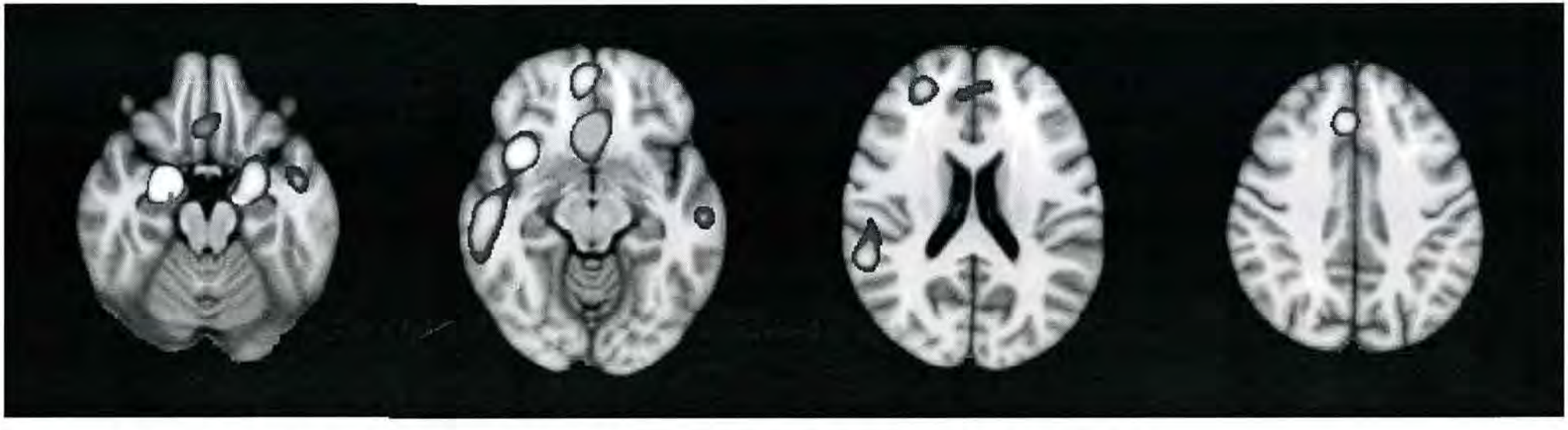


Whole brain rs-MEG source-amplitude images averaged from 41healthy subjects in MNI-152 atlas coordinates from Fast-VESTAL in alpha (first row), beta (second row), gamma (third row), and low-frequency (delta plus theta, fourth row) bands.

Huang et al., Neuroimage 2014 84: 585-606

MEG detects brain abnormalities in PTSD

Gamma-band hyper- and hypo-activities in PTSD vs healthy controls (t-test, corrected p<.01)



- **Abnormal activity in "Fear Circuit" of the brain in PTSD**
- Hyper-activity: L-R Amygdala; Anterior cingulate; R insular cortex; R frontal; R temporal lobe
- Hypo-activity: inferior medial frontal lobe
- **Both mTBI and PTSD are visible, each showing specific abnormalities in different parts of the brain**

Clinical Implications

- Having a TBI event increases likelihood of PTSD, with implications for screening and treatment

- We don't yet know what the mechanism for the increase in PTSD following deployment TBI
  - The emotional salience of the event(s) contiguous with TBI?
  - Structural and functional brain changes following TBI?

- Newer imaging methods can help with diagnosis by making the previously invisible - TBI and PTSD - visible

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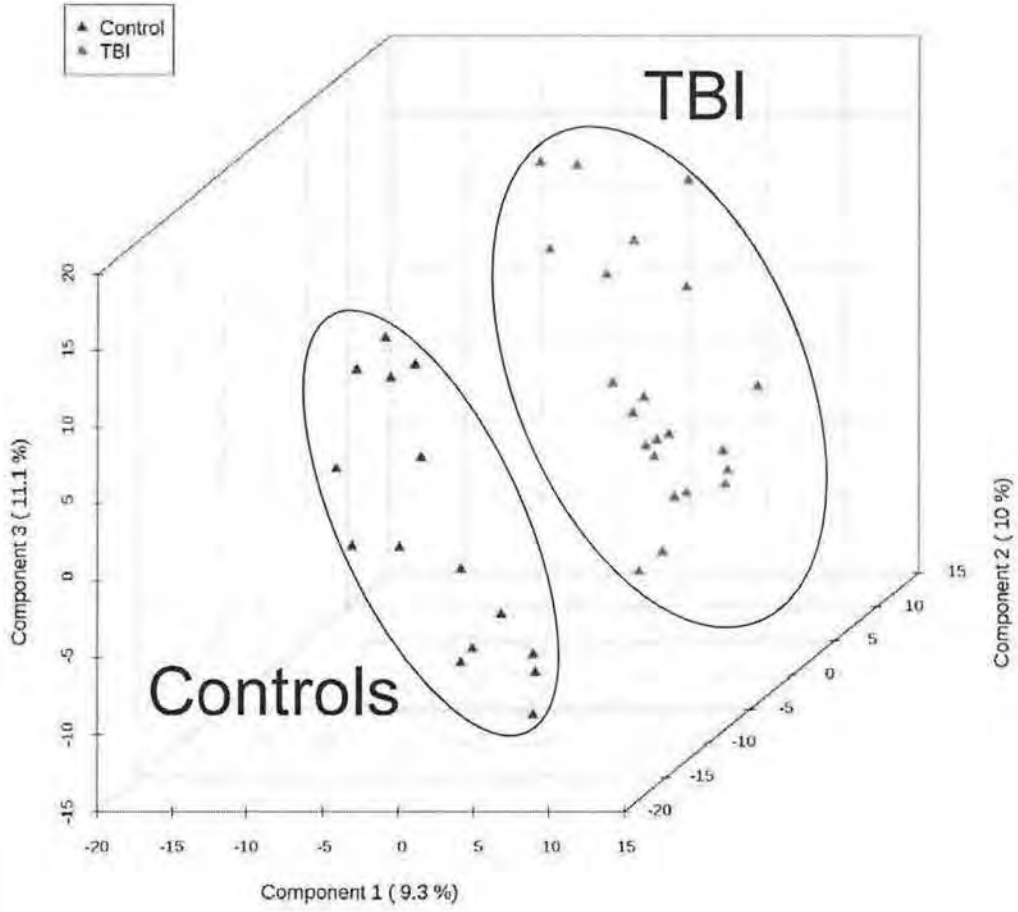
Metabolomic Diagnosis of TBI



24 Metabolites were diagnostic

Metabolites to be validated against other half of the group

N = 22 TBI, 16 Controls

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Ongoing Work

- Through an ongoing MEG study and analysis of already collected data from MRS and MRS-II we are working to:
  - Better understand the "dose" effects of head injury (as measured by blast sensors, DARPA) on TBI abnormalities observed on MEG images
  - Understand how TBI impacts vulnerability to PTSD, as indexed by emotional learning
  - Carefully assess emotional and cognitive measures as they relate to imaging findings
  - Pursue a reliable blood biomarker

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Contributors and Collaborators

MRS-II Investigators

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- Dewleen Baker M.D. VA & UCSD
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- Richard Hauger M.D., VA & UCSD
- Sharon Nichols, Ph.D. UCSD

- MRS Research Team

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- **Jay Churchill, NIMH ARMY STARRS program officer and HQMC & MRS-II Liaison with NIMH**

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Title: Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in Active Duty Marines

Article Type: SI: Biomarkers in the military

Keywords: Extinction; Fear; PTSD; Anxiety; Military; Fear inhibition; Safety Signal; Startle

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Order of Authors: Dean Acheson, PhD; Mark Geyer; Dewleen Baker; Caroline Nievergelt; Kate Yurgil; Victoria Risbrough

Abstract: Posttraumatic Stress Disorder (PTSD) is a major public health concern, especially given the recent wars in Iraq and Afghanistan. Nevertheless, despite a sharp increase in the incidence of psychiatric disorders in returning veterans, empirically based prevention strategies are still lacking. To develop effective prevention and treatment strategies, it is necessary to understand the underlying biological mechanisms contributing to PTSD and other trauma related symptoms. The "Marine Resiliency Study II" (MRS-II; Oct 2011-Oct 2013) Neurocognition project is a longitudinal investigation of neurocognitive performance in Marines deployed to Afghanistan. As part of this investigation, 1,195 Marines and Navy corpsmen underwent a fear conditioning and extinction paradigm and psychiatric symptom assessment prior to deployment. The current study assesses 1) the effectiveness of the fear potentiated startle paradigm in producing fear learning and extinction, and 2) the association of performance in the paradigm with baseline psychiatric symptom classes (Healthy, PTSD symptoms, Anxiety symptoms, and Depression symptoms). Results suggest that the task was effective in producing differential fear learning and fear extinction in this cohort. Further, distinct patterns emerged differentiating the PTSD and Anxiety symptom classes from both Healthy and Depression classes. In the fear acquisition phase, the PTSD group was the only group to show deficient discrimination between the conditioned stimulus (CS+) and safety cue (CS-), exhibiting larger startle responses during the safety cue compared to the healthy group. During extinction learning, the PTSD group showed significantly less reduction in their CS+ responding over time compared to the healthy group, as well as reduced extinction of self-reported anxiety to the CS+ by the end of the extinction session. Conversely, the Anxiety symptom group showed normal safety signal discrimination and extinction of conditioned fear, but exhibited increased baseline startle reactivity and potentiated startle to CS+, as well as higher self reported anxiety to both cues. The Depression symptom group showed similar physiological and self-report measures as the healthy group. These data are consistent with the idea that safety signal discrimination is a relatively specific marker of PTSD symptoms compared to general anxiety and depression symptoms. Further research is needed to determine if deficits in fear inhibition vs. exaggerated fear responding are separate biological "domains" across anxiety disorders that may predict differential biological mechanisms and possibly treatment needs. Future longitudinal analyses will examine whether poor learning of safety signals provides a marker of vulnerability to develop PTSD or is specific to symptom state.

*Cover Letter

UNIVERSITY OF CALIFORNIA, SAN DIEGO UCSD

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April 14, 2014

Dear Drs Vermeeten and Yehuda:

Attached please find a manuscript titled "Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in Active Duty Marines " for consideration for your special issue on *Biomarkers in the Military*. This manuscript describes findings that in a sample of >1000 Active Duty Marines, disruption in "safety signal" learning is a specific behavioral marker of PTSD symptoms compared to depression or anxiety symptoms. This manuscript shows for the first time, a direct comparison of groups with relatively specific PTSD, anxiety or depression symptoms. We found these groups exhibit differential patterns of fear conditioning and extinction, with PTSD and anxiety symptom groups showing reduced fear inhibition and increased fear responding respectively. These data support the use of objective behavioral markers that can help delineate differential biological mechanisms underlying these symptom classes. We hope that you will find this manuscript suitable for publication in *Psychoneuroendocrinology*.

Best regards,

Victoria Risbrough

*Abstract

Posttraumatic Stress Disorder (PTSD) is a major public health concern, especially given the recent wars in Iraq and Afghanistan. Nevertheless, despite a sharp increase in the incidence of psychiatric disorders in returning veterans, empirically based prevention strategies are still lacking. To develop effective prevention and treatment strategies, it is necessary to understand the underlying biological mechanisms contributing to PTSD and other trauma related symptoms. The "Marine Resiliency Study II" (MRS-II; Oct 2011-Oct 2013) Neurocognition project is a longitudinal investigation of neurocognitive performance in Marines deployed to Afghanistan. As part of this investigation, 1,195 Marines and Navy corpsmen underwent a fear conditioning and extinction paradigm and psychiatric symptom assessment prior to deployment. The current study assesses 1) the effectiveness of the fear potentiated startle paradigm in producing fear learning and extinction, and 2) the association of performance in the paradigm with baseline psychiatric symptom classes (Healthy, PTSD symptoms, Anxiety symptoms, and Depression symptoms). Results suggest that the task was effective in producing differential fear learning and fear extinction in this cohort. Further, distinct patterns emerged differentiating the PTSD and Anxiety symptom classes from both Healthy and Depression classes. In the fear acquisition phase, the PTSD group was the only group to show deficient discrimination between the conditioned stimulus (CS+) and safety cue (CS-), exhibiting larger startle responses during the safety cue compared to the healthy group. During extinction learning, the PTSD group showed significantly less reduction in their CS+ responding over time compared to the healthy group, as well as reduced extinction of self-reported anxiety to the CS+ by the end of the extinction session. Conversely, the Anxiety symptom group showed normal safety signal discrimination and extinction of conditioned fear, but exhibited increased baseline startle reactivity and potentiated startle to CS+, as well as higher self reported anxiety to both cues. The Depression

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Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in Active Duty Marines

Acheson, D.T.[1], Geyer, M.A.[1,3], Baker, D.G.[1,2], Nievergelt, C.M.[1,2], Yurgil, K.[2], Risbrough, V.B.*[1,2], & MRS-II Team

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Key Words: Extinction; Fear; PTSD; Anxiety; Military; Fear inhibition; Safety Signal; Startle

Running Title: Fear conditioning across PTSD, anxiety and depression symptom groups

Abstract

Posttraumatic Stress Disorder (PTSD) is a major public health concern, especially given the recent wars in Iraq and Afghanistan. Nevertheless, despite a sharp increase in the incidence of psychiatric disorders in returning veterans, empirically based prevention strategies are still lacking. To develop effective prevention and treatment strategies, it is necessary to understand the underlying biological mechanisms contributing to PTSD and other trauma related symptoms. The "Marine Resiliency Study II" (MRS-II; Oct 2011-Oct 2013) Neurocognition project is a longitudinal investigation of neurocognitive performance in Marines deployed to Afghanistan. As part of this investigation, 1,195 Marines and Navy corpsmen underwent a fear conditioning and extinction paradigm and psychiatric symptom assessment prior to deployment. The current study assesses 1) the effectiveness of the fear potentiated startle paradigm in producing fear learning and extinction, and 2) the association of performance in the paradigm with baseline psychiatric symptom classes (Healthy, PTSD symptoms, Anxiety symptoms, and Depression symptoms). Results suggest that the task was effective in producing differential fear learning and fear extinction in this cohort. Further, distinct patterns emerged differentiating the PTSD and Anxiety symptom classes from both Healthy and Depression classes. In the fear acquisition phase, the PTSD group was the only group to show deficient discrimination between the conditioned stimulus (CS+) and safety cue (CS-), exhibiting larger startle responses during the safety cue compared to the healthy group. During extinction learning, the PTSD group showed significantly less reduction in their CS+ responding over time compared to the healthy group, as well as reduced extinction of self-reported anxiety to the CS+ by the end of the extinction session.

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Introduction

Posttraumatic Stress Disorder (PTSD) is a major public health concern among current and former military members, including those who have recently experienced combat in Iraq and Afghanistan (Baker et al., 2012). For instance, since these wars began in 2001, the incidence of psychiatric disorders among active-duty service members has increased by 62%, with an increase of 656% for PTSD and 226% for anxiety disorders. In addition, the cost to the Department of Defense (DoD) for treating these service members doubled between 2007 and 2012 (Blakely & Jansen, 2013 Congressional Research Service Report). The Department of Veterans Affairs (VA) and society at large will continue to bear the cost of treating service members with chronic psychiatric issues long after these individuals are discharged from the military. According to a recent report by the Institute of Medicine, DoD prevention efforts are hampered by an insufficient empirical base (National Research Council, 2014). Identifying the underlying biological mechanisms of PTSD from other stress-related disorders is a key step in developing an evidence base on which to design more effective prevention and treatment efforts.

The "Marine Resiliency Study II" (MRS-II; Oct 2011-Oct 2013) Neurocognition project is a longitudinal investigation of neurocognitive performance in Marines deployed to Afghanistan. Similar to the original MRS (Baker et al. 2012), Marines were assessed in a 3.5 hr test battery in which clinical assessment, self-report, and biological assays are combined with comprehensive neurocognitive assessments once before deployment and then again 3-6 months after deployment. The purpose of MRS-II is to discriminate between biological markers that predict risk/resiliency for development of combat-stress

related disorders and markers associated specifically with symptom state. Here we focus on one aspect of these assessments, measurement of fear conditioning and extinction learning.

Increased responses to conditioned fear cues and reduced ability to inhibit these responses are well-known features of PTSD in civilian and combat-veteran populations (for review see VanElzakker et al., 2013). Reduced ability to inhibit fear has recently been suggested to be a potential "biomarker" specific to PTSD, with PTSD subjects exhibiting poor learning of safety signals (cues that predict absence of threat) compared to depressed subjects (Jovanovic & Norrholm, 2011; Jovanovic et al., 2009; 2010). Studies in high trait anxious participants or other anxiety disorders are inconsistent, showing either normal or reduced fear inhibition as measured by safety signal learning (Kindt & Soeter, 2014; Gazendam et al., 2013; Lissek et al., 2009). Reduced inhibition in PTSD patients is thought to reflect disruption of frontal cortical and hippocampal circuits to inhibit amygdala activation and concomitant fear responses (Admon et al., 2013; Acheson et al. 2012). However, increased fear responding to conditioned cues, aversive contexts, or overgeneralization of fear responses are shown across multiple anxiety disorders and thus may reflect biological processes that are shared across disorders (McTeague et al., 2012; Lissek et al., 2013; Grillon, 1998). Results are less clear however for depression, with reports of lower, normal, and higher aversive responding or fear conditioning (McTeague et al., 2012; Grillon et al., 2013; Robinson et al., 2012; Jovanovic et al, 2010) depending on the type of conditioned cues and aversive stimuli. Heightened fear responding may be due to increased amygdala, extended amygdala, and/or dorsal anterior cingulate activity in these disorders (Admon, 2013;

Grillon, 2008). Understanding the differential patterns of fear conditioning and inhibition between symptom types will help identify specific endophenotypes for further biological interrogation across stress-related disorders (Cuthbert & Kozak, 2013; McTeague et al., 2012; Admon et al., 2013). Given that MRS-II is a longitudinal study, we will ultimately be able to determine in future analyses if these putatively differential phenotypes are vulnerability factors or related specifically to symptom state after trauma.

To test the hypothesis that PTSD, depression, and general anxiety symptoms may reflect distinct biological mechanisms and subsequent differential patterns of fear conditioning and inhibition abnormalities, we used a cross-sectional design to directly compare fear conditioning and extinction across participants endorsing symptoms of general anxiety, depression, and PTSD at pre-deployment. We used the fear potentiated startle (FPS) paradigm established by Norrholm and colleagues (2006), as this paradigm is sensitive to both the reduced fear inhibition (i.e. safety signal learning and extinction) and increased fear conditioning described in PTSD patients (Norrholm et al., 2011). This protocol uses an aversive air-puff as the unconditioned aversive stimulus. Though other fear conditioning paradigms have used aversive electrical shock as the unconditioned stimulus (i.e., Milad et al., 2007), we chose to use air puff for a number of reasons. One, use of an air puff increased the feasibility of testing such a large active duty population in a time-limited manner as it does not require initial "customization" of shock stimuli. Lack of required customization reduced setup time as well as technical difficulty. Two, we anticipated that shock stimuli would be less acceptable to study participants and to local and military institutional review boards given the special population status of active duty military. Third, this protocol uses startle reactivity as the operational measure of

conditioned fear, a cross species measure of fear conditioning for translational applications in animal models, and which may be more sensitive to "automatic" or implicit fear learning compared to other measures such as skin conductance (Sevenster et al., 2014; Glover et al., 2011).

Methods

Participants

1,195 infantry Marines and Navy Corpsmen enrolled in a longitudinal study of the health effects of deployment to Afghanistan. Two separate infantry battalions were studied, with data collection occurring 1-2 mo prior to deployment. At the time of this collection period all Marine infantry were male, thus females did not participate. This study was approved by the institutional review boards of the University of California San Diego, VA San Diego Research Service, and the Naval Health Research Center. Written informed consent was obtained from all participants.

Fear Conditioning and Extinction Procedure

Apparatus: Startle pulses (108 dB, 40 ms) were delivered using a San Diego Instruments (SDI, San Diego, CA, USA) SR-HLAB Electromyography (EMG) system. Sound levels were measured using continuous tones calibrated with a Quest Sound Level Meter on the A scale, coupled to the headphones with an artificial ear. The air puff was set at 250 psi and delivered via a plastic tube positioned 2.5 cm from the center of the throat. Air-puff onset was controlled by a solenoid system triggered by the same Acer laptop computer that controlled the startle stimuli. Conditioned stimuli were presented via E-Prime software (Psychology Software Tools, Inc., Sharpsburg, PA, USA) run on a

Dell desktop computer with a 48 cm monitor positioned directly in front of the participant. Presentation of the stimuli by the E-Prime software was triggered by signals from the EMG system to control synchronization of conditioned, startle, and air-puff stimuli.

Eyeblink EMG responses were recorded via Ag/Ag 3M Red Dot electrodes placed at the *orbicularis oculi* muscles at the left eye connected to the SDI SR-HLAB EMG system and Acer laptop computer (Acheson et al., 2013; 2012). A reference electrode was placed at the mastoid bone behind the left ear. Before electrode placement, skin was cleaned with an alcohol swab and gently exfoliated with 3M electrode prep tape. All electrode resistances were <10 kΩ. EMG data were recorded at a sampling rate of 1 KHz, amplified (0.5 mV electrode input was amplified to 2500 mV signal output), band-pass filtered (100-1000 Hz), rectified, and then smoothed with a 5-point rolling average. Expectancy responses were recorded on a trial-by-trial basis via the participant"s responses on a key pad linked to E-Prime software. Additional self-report responses were recorded at the end of each experimental phase via the same keypad.

Eyeblink data were scored via SR-HLAB EMG Utilities software as previously described (Acheson et al., 2012). In brief, eyeblink responses were examined on a trial by trial basis at a window starting 100 ms before the startle pulse and ending 200 ms after the pulse. Only responses that peaked within 100 ms of pulse onset were scored as a startle response. Trials in which excessive baseline noise or artifact obscured the startle response were removed (2.1% of trials) and replaced with an imputed value based on the average of the immediately preceding and following trials.

Fear Conditioning and Extinction task: The fear conditioning and extinction protocol consisted of two discrete testing sessions or "phases": Acquisition and Extinction. Before the acquisition phase the participants were instructed that one of the colored symbols predicted when the airpuff would appear. Each phase began with 6 startle pulses presented in the absence of any other stimuli to stabilize startle responding. The Acquisition phase consisted of 8 6-sec presentations of the conditioned stimulus (CS+; either a blue or yellow circle or square, balanced across subjects) that was paired with the air puff in 75% contingency, 8 6-second presentations of a non-reinforced conditioned stimulus (CS-; also either a blue or yellow circle or square) that was never paired with the air puff, and 8 presentations of the startle stimulus in the absence of any stimuli (noise alone or "NA" trial) which served as a measure of baseline startle across the phase. The CS+ and air puff co-terminated on reinforced trials. Startle pulses were presented approximately 4 sec following CS+ or CS- onset. The stimuli serving as CS+ and CS- (blue or yellow circles or squares) were randomly assigned across participants. Contingency awareness was measured using a numbered keypad to report at each CS+ and CS- trial whether or not they expected to receive the air puff. Participants responded with a "1" if they expected the air puff, "2" if they were unsure, and "3" if they did not expect the air puff. After the acquisition phase, contingency awareness was again assessed via a questionnaire asking participants which stimulus predicted the shock. Self-reported anxiety during the cues was also measured at this time, as was the subjective aversiveness of the air-puff stimuli.

After completing the Acquisition phase, participants were asked to sit quietly for 5 min before beginning the Extinction phase. Before the extinction phase began, the

subjects were told to "remember what the learned" in the previous session. The Extinction phase consisted of 16 presentations of each stimulus type (CS+, CS-, and NA). No air puffs were presented during this phase. Presentations of startle pulses were delivered and ratings of air-puff expectancy were collected in the same fashion as in the Acquisition phase. After this phase, participants again rated their level of anxiety during the cues. After these ratings were made, participants were disconnected from the apparatus and went on to other assessment stations (see Baker et al. 2012 for full details of Marine Resiliency Study assessment battery).

Assessment of Psychiatric Symptoms

Posttraumatic Stress Disorder: Post-traumatic stress symptoms were assessed using a structured diagnostic interview, the Clinician Administered PTSD Scale (CAPS; Blake et al., 1995). CAPS total scores can range from 0 to 136 and can be used as a measure of PTS symptom severity. PTSD symptom group membership was defined using the partial PTSD criteria articulated by Stein and colleagues (Stein et al., 1997). Partial PTSD criteria were chosen due to the relative psychological health of an active duty Marine cohort. Criteria for assignment to the PTSD symptom group were the presence of at least 1 B symptom, 2 C symptoms, and 2 D symptoms, with minimum frequency ratings of 1 and minimum intensity ratings of 2. Inter-rater reliability in MRS was high for both the CAPS total score (Intraclass correlation coefficient = .99) and for PTSD diagnosis (Kappa = .714).

Anxiety: Assignment to the anxiety symptoms group was defined as scoring in the Moderate to Severe range (> 15) on the Beck Anxiety Inventory (BAI; Beck & Steer,

1993). The BAI is a reliable measure of general anxiety symptoms present within the past week, and discriminates between anxiety vs. depressive symptoms fairly well (Clark et al., 1994).

Depression: Assignment to the depression symptoms group was defined as scoring in the Moderate to Severe range (> 19) on the Beck Depression Inventory 2 (BDI-2; Beck et al., 1996). The BDI-2 measures the presence of depressive symptoms within the past 2 weeks.

Trauma History: The Life Events Checklist (LEC; Gray et al., 2004) was used to assess previous trauma history. The LEC evaluates the participant"s experience of a wide range of traumatic events and further assesses whether the event directly happened to the individual, the individual witnessed the event happening to others, or whether the even was learned about second-hand. The LEC score reported here was calculated by summing all of the items scored as "happened to me" and/or "witnessed it".

Data Analysis

Final Sample: Of the original 1,195 Marines and Corpsmen who underwent the fear conditioning and extinction protocol, data on 21 were rendered unusable due to technical difficulties during testing. An additional 125 (10.6% of the remaining sample) were excluded from the analysis because they failed to show a CS+ response greater than baseline during the last half of the Acquisition phase. This failure to potentiate above baseline suggested that the air puff was ineffective in inducing fear in these subjects that would be sufficient to support learning in these participants. Further, 35 subjects met our cutoffs for more than one symptom group and were excluded from the analysis. This

approach was taken to enable comparison of relatively "pure" symptom classes on fear conditioning and extinction phenotypes. See supplemental materials Table S1. for demographic data on these excluded subjects. The remaining 1,014 subjects were included in all analyses.

Startle: Startle data for the Acquisition and Extinction phases were analyzed as previously described in Acheson et al. (2013) by averaging responses to each stimulus type into blocks of two trials. Within each block, the NA averages were subtracted from the CS+ and CS- averages to adjust for changes in baseline startle across the session. Thus, each CS+ and CS- block represented startle above baseline for that block (e.g., (CS+) – (NA)). Thus there were 4 blocks for the CS+ and CS- during the Acquisition phase, and 8 blocks for the CS+ and CS- for the Extinction phase.

To compare acquisition across symptom groups, the analysis was simplified by averaging the last two blocks of the session across both CS types to create a measure of responding over the last half of the acquisition phase. To assess function of the task, Acquisition phase data were initially analyzed within the healthy group only using a repeated-measures ANOVA to assess differences in response to each CS type. To assess differences by symptom group, a 2 (CS type) x 4 (symptom group) mixed ANOVA was conducted on the entire sample. Significant interactions were followed up with alpha-adjusted post-hoc tests to assess Cue response differences within each symptom group. To assess symptom group differences in baseline startle, a one-way ANOVA, with appropriate post-hoc tests, was conducted on the average NA trial response across the last half of the extinction phase.

Extinction phase data were analyzed by computing a measure of "% conditioned fear". This score is similar to the "extinction retention index" originated by Milad and colleagues (2007; 2008) in their studies of fear extinction memory recall, which use a normalization approach to reduce confounds of differences in fear conditioning on measurement of extinction. For each subject, the maximal CS+ response during the acquisition phase is identified. A % conditioned fear is then calculated for each of the 8 extinction blocks using the following equation: 100*(CS+ response on extinction block/maximum response across acquisition blocks). For simplicity of presentation and analysis, these scores were further averaged into 4 Extinction blocks consisting of 4 trials each. The first block, Early Extinction, consisted of the first 4 trials of the phase, Mid Extinction 1 trials 5-8, Mid Extinction 2 trials 9-12, and Late Extinction trial 13-16. To assess function of the task, Extinction phase data were initially analyzed within the healthy group only using a repeated-measures ANOVA to assess decrease in responding across the phase. To assess differences by symptom group, a 4 (symptom group) x 4 (Extinction Block) mixed ANOVA was conducted on the entire sample. To assess symptom group differences in baseline startle response during the extinction phase, a 4 (symptom group) x 4 (Extinction Block) mixed ANOVA, with appropriate post-hoc tests, was conducted on the NA responses averaged into blocks analogous to those above.

Expectancy and Self-Report: Expectancy responses were re-coded as: expect air puff = 1, unsure = 0, do not expect air puff = -1. Expectancy responses over the last half of the Acquisition phase (4 trials/stimulus type) were averaged together as with the startle data. ANOVAs were applied to assess both task effectiveness and differences by symptom group in the same manner as with the startle responses.

Expectancy responses during the extinction phase were analyzed by trial, including the last 4 trials of the Acquisition phase (20 total trials). Task effectiveness was assessed using a repeated-measures ANOVA on the healthy group only. A 4 (symptom group) x 20 (Trial) mixed ANOVA was used to assess differences by symptom group across the entire sample.

To assess task effectiveness on self-reported anxiety, CS type differences on post-phase questionnaires were analyzed using repeated measures ANOVA on the healthy group alone. A 2 (CS type) x 4 (symptom group) mixed ANOVA was used to assess differences across symptom groups. Task effectiveness in assessing change across phase in self-reported anxiety was assessed using a repeated-measures ANOVA in the healthy group only. Differences across phase by symptom group were assessed with 4 (symptom group) x 2 (Phase) mixed ANOVA on the entire sample. In all analyses, significant interactions were followed up with two-tailed Tukey post-hoc tests.

Results

Demographics

Sample demographics are displayed in Table 1. There were no differences across symptom groups on any demographic variable. Differences between symptom groups did emerge on the LEC [$F(3,1010)=9.03$, $p<.0001$, partial $\eta^2=.03$], such that all symptom groups reported more trauma experience relative to healthy controls ($ps<.04$). However, the symptom groups did not differ from one another. Two subjects were taking psychiatric medication for reasons other than smoking cessation or sleep (1 in the PTSD symptom group and 1 in the anxiety symptom group). Both of those subjects reported taking fluoxetine at unknown dosages. As expected from our selection criteria, the

symptom groups had significantly higher scores on their respective assessment measures relative to the other groups (Table 1; omnibus tests $F(3,1010)>129.55$, $ps<.0001$; $ps<.05$ for comparisons vs reference group). All symptom groups had higher levels of PTSD, anxiety and depression symptoms compared to controls healthy controls ($ps<.05$).

Overall Task Effectiveness

Acquisition

Startle: As expected, startle responses during the Acquisition phase showed a significant effect of Cue type, with the CS+ response being elevated relative to the CS-, indicating successful differential fear conditioning [Figure 1A, $F(1,918)=475.14$, $p<.0001$, partial $\eta^2=.34$].

Expectancy and Self-Report: For expectancy ratings, participants correctly identified the CS+ as predictive of the shock [Figure 2A; $F(1,913)=3916.39$, $p<.0001$, partial $\eta^2=.811$]. On a 1 (expect air puff) to -1 (do not expect air puff) scale, participants averaged a 0.59 rating for the CS+ and a -0.78 rating for the CS-.

On the post-phase questionnaire, 88.9% of participants correctly identified the CS+ as predictive of the air puff. 6.7% of participants were not sure which CS predicted the air puff, and 3.1% misidentified the CS- as predictive of the air puff. Overall, participants assigned the air puff an average aversiveness rating of 2.31 out of 5 (SD = 1.02). Participants rated higher levels of subjective anxiety in the presence of the CS+ relative to the CS-, again indicative of differential fear conditioning [Figure 3A; $F(1,911)=1298.43$, $p<.0001$, partial $\eta^2=.588$].

Extinction

Startle: As expected, percentage of conditioned fear (normalized to the fear levels displayed in the acquisition phase) decreased significantly across the phase, demonstrating successful fear extinction [Figure 2A; $F(3,2751)=182.87$, $p<.0001$, partial $\eta^2=.166$].

Expectancy and Self-Report: Expectancy ratings to the CS+ decreased significantly across the late Acquisition and Extinction phases [Figure 2B; $F(19,16682)=573.56$, $p<.0001$, partial $\eta^2=.395$]. From the Acquisition to Extinction phases, post-phase ratings of anxiety to the CS+ decreased significantly [Figure 3B; $F(1,902)=529.15$, $p<.0001$, partial $\eta^2=.37$].

Comparison of Task Performance between Psychiatric Symptom Groups

Acquisition

Baseline Startle: There was a significant difference between symptom groups in average baseline startle during the last half of the acquisition phase [$F(3, 1010)=3.05$, $p<.03$, partial $\eta^2=.009$], such that the Anxiety group had a higher magnitude of startle relative to healthy controls ($p<.009$). No other symptom group differed from healthy controls.

Startle Potentiation: When participants meeting criteria for inclusion in a symptom group were examined, a significant symptom group x Cue type interaction emerged [Figure 1A; $F(3,1005)=3.4$, $p<.02$, partial $\eta^2=.01$]. Post-hoc tests revealed that responding to the CS+ was significantly higher than responses to the CS- for the healthy, anxious, and depressed symptom groups ($ps<.001$), but not for the PTSD group ($p<.09$)

suggesting reduced differential fear conditioning in the PTSD group. This deficit in differential conditioning was driven by higher CS- responses in the PTSD group relative to the healthy group ($p<.004$). In contrast, the Anxiety group exhibited a trend for increased CS+ responding ($p<0.06$) and no significant differences in CS- responses compared to healthy controls. Maximum CS+ responding was also calculated across the groups, and the anxiety symptom group showed significantly larger maximum CS+ responses compared to the healthy group [Supplemental Figure 1; $F(3,1010)=2.73$, $p<.05$, partial $\eta^2=.008$; anxiety vs. healthy $p<.02$]

Expectancy and Self-Report: For expectancy ratings, there was no symptom group x Cue type interaction [Figure 2A; $F(3,1000)=1.62$, ns], nor was there an overall effect of symptom group [$F(3,1000)<1.0$, ns]. For self-reported anxiety, there was a significant effect of symptom group [Figure 3A; $F(3,997)=5.78$, $p<.001$, partial $\eta^2=.017$] with anxious subjects reporting higher levels of anxiety in response to both cues ($p<.001$). There was no symptom group x Cue type interaction [$F(3,997)=1.65$, ns].

Extinction

Baseline Startle: There was a trend toward differential responding between symptom groups across the extinction phase [$F(3, 1010)=2.09$, $p<.1$, partial $\eta^2=.006$], again with the Anxiety group trending toward higher response relative to healthy controls ($p<.1$).

Startle Potentiation: A significant main effect of symptom group was apparent on %conditioned fear during the extinction phase [$F(3,1005)=3.05$, $p<.03$, partial $\eta^2=.009$], such that the PTSD group maintained a higher level of conditioned fear across the entire

session compared to the healthy controls ($p<.006$). There was also a trend for a block X symptom group interaction [Figure 2A; $F(9,3015)=1.66$, $p<.1$, partial $\eta^2=.005$]. Exploratory post-hoc analyses at each block showed that the PTSD group maintained a higher level of conditioned fear relative to healthy controls at both the Mid Extinction 2 and Late Extinction blocks ($ps<.05$). The Anxiety group showed a trend toward higher responding relative to controls during Mid Extinction 1 ($p<.07$), however this trend was not apparent at the later Extinction blocks. The Depression group did not differ from healthy controls.

Expectancy and Self-Report: Expectancy ratings to the CS+ did not vary by symptom group across the phase [Figure 2B; $F(45, 14505)=1.33$, ns], nor was there a main effect of symptom group [$F(3,967) < 1.0$, ns]. For self-reported anxiety, there were significant differences in change across phases by symptom group [Figure 3B; $F(3,988)=4.24$, $p<.01$, partial $\eta^2=.013$], such that all groups showed significant reductions across phase ($ps < .05$) with the exception of the PTSD group. The PTSD and Anxiety groups had higher responses to the CS+ during the extinction phase relative to the healthy group ($ps < .02$). In addition, there was a significant main effect of symptom group, with the Anxiety group showing higher ratings overall relative to the Healthy group [$F(3,988)=5.12$, $p<.002$, partial $\eta^2=.015$].

Discussion

As expected, the conditioning paradigm was effective in producing conditioned fear learning and subsequent extinction learning in our active-duty Marine and Navy volunteers. Psychiatrically healthy participants acquired differential fear-potentiated

startle and self-reported anxiety responses to the CS+ vs. the CS- and showed contingency awareness (expectancy ratings). Across the extinction phase, when the air puff was absent, responses to the CS+ decreased in terms of both potentiated startle and self-reported anxiety. Expectancy ratings showed intact contingency learning across extinction as well. Successful learning in this paradigm enables comparisons to be made in the learning patterns among the various psychiatric symptom groups.

Differential patterns of learning performance emerged between psychiatric symptom groups. The PTSD group was unique in failing to show a differential potentiated startle response to CS+ and CS- at the end of fear acquisition. This failure was due to PTSD subjects maintaining a relatively high startle response to the CS-. The observation of high startle responses to the CS- is in line with existing research showing that individuals with PTSD have difficulty learning to inhibit startle responses in the presence of a safety signal (Jovanovic et al., 2009; 2010). Though not explicitly termed "safety signal" in the current paradigm, presentation of the CS- effectively signals the absence of the air puff, or safety. Interestingly, the participants in the PTSD group showed intact contingency awareness in the expectancy ratings, as well as intact discrimination learning as assessed by self-reported anxiety. These findings suggest a "disconnect" between the participant"s explicit experience and automatic physiological responses to the safety cue (i.e. potentiated startle).

Across the extinction phase, the PTSD symptom group maintained potentiated startle to the CS+ overall relative to the healthy group. The finding that conditioned fear responses were maintained throughout extinction supports existing research suggesting a disruption in fear extinction learning and recall in PTSD subjects relative to healthy

controls (Norrholm et al., 2011; Milad et al., 2008; Wessa & Flor, 2007; Orr et al., 2000, Peri et al., 2000). This greater maintenance of conditioned fear was also apparent in the self-report of anxiety in response to the CS+, which remained relatively unchanged in the PTSD group after extinction training, unlike the other groups. Again, the PTSD group showed normal explicit learning that the CS+ no longer predicted the US (as evidenced by the expectancy ratings across the extinction session), further supporting a disconnect between explicit contingency awareness and fear expression. Thus the current findings of deficient inhibition of potentiated startle to a safety cue and reduced extinction of physiological and emotional fear responses in the presence of intact contingency awareness supports the theory that PTSD is characterized by a failure to inhibit automatic, physiological fear responses. This failure of inhibition is observed even though the subject is explicitly aware of a lack of threat or danger.

The anxiety symptom group showed significantly higher baseline startle responding and higher CS+ potentiation compared to the healthy group. This group also reported significantly higher anxiety to both CS+ and CS- after acquisition relative to the healthy group. The finding that CS+/- discrimination is normal in participants with high generalized anxiety symptoms is in line with other report that high trait anxiety participants exhibit normal CS+/CS- discrimination (Kindt and Soeter, 2014; Gazendam et al, 2013). The present findings of higher self-reported anxiety to the conditioned cues are also in line with past reports using a similar protocol (Gazendam et al, 2013). During extinction training, the anxiety symptom group successfully extinguished both potentiated startle and US expectancy to the CS+. They also successfully extinguished self-reported anxiety to the CS+, however overall responding remained high compared to

the other groups. Taken together, this pattern of results is suggestive of greater explicit anxiety responses during aversive anticipation in this group while fear inhibition and discrimination processes are relatively normal.

The depression symptom group showed response patterns in all measures that were indistinguishable from healthy controls. The normal fear inhibition and potentiated startle in the depression group as assessed by safety signal learning and extinction is in line with previous studies (Jovanovic et al., 2010; 2012). The present results differ however from a recent study in major depression patients in a task which incorporates both predictable and unpredictable aversive stimuli (Grillon et al., 2013). In this task, MDD patients exhibited higher baseline startle reactivity as well as greater potentiation during the cue that was predictive (100% contingency) of an aversive event. The increased startle potentiation was associated with symptom chronicity as well as severity. The different results across this study and the present study are unlikely due to differences in symptom severity (mean BDI 26 vs. 29 for present and previous studies, respectively) or treatment (both studies used unmedicated participants). It is possible that the difference between the Grillon et al. study and the present study are due to differences in the chronicity of symptoms, gender demographics (mixed vs. all male sample respectively) and comorbid anxiety (high vs. relatively low respectively). The lack of significant differences in the present study must also be interpreted with caution given the relatively small sample size in this group (N=12).

The present results suggest differential performance between PTSD and anxiety symptom groups, with general anxiety symptoms being more associated with exaggerated fear responses and PTSD symptoms being specifically associated with a failure to

appropriately inhibit fear responses to safety signals and reduced extinction. This differential pattern of results is suggestive of differences at the neurocircuit level. The higher overall responding in the anxiety symptom group may reflect hyperactivity in emotion-generating limbic circuits, consistent with the neuroimaging evidence for heightened amygdala activation to negative provocation in subjects with generalized anxiety (ie, Rauch et al., 2003). While PTSD has also been associated with limbic system hyperactivity (Shin et al., 2006), neuroimaging studies have shown more pronounced findings of hypoactivation in structures responsible for inhibition of the limbic system, specifically the medial prefrontal cortex (mPFC) and the rostral and dorsal regions of the anterior cingulate cortex (Etkin & Wager, 2007). Further, Milad and colleagues (2007; 2008) have demonstrated that individuals with PTSD exhibit reduced ability to recall fear extinction (or fear inhibition) 24 hours after initial learning, an ability that is dependent upon mPFC activation. Reduced activity of ventromedial prefrontal cortex is also associated with increased potentiation to CS- and reduced extinction of CS+ (Jovanovic et al 2013). Thus this pattern of hypoactivation in fear inhibition circuits may be reflected in the current results of relatively normal magnitude of fear responses but poor safety-signal learning and reduced extinction in PTSD symptom groups. The present findings also raise the possibility that this task could identify, via differential patterns of response (exaggerated fear response vs. impaired fear inhibition), those who are neurobiologically at risk for developing a certain class of pathology post-trauma. Previous research has suggested that impaired fear extinction may be a marker for increased risk of developing PTSD following a trauma (Guthrie & Bryant, 2006; Pole,

2009; Lommen, 2013). Future studies may examine whether these phenotypes predict differential treatment responses to pharmacological or behavioral therapies.

Some limitations of the current study must be acknowledged. First, the paradigm was not effective in producing fear-potentiated startle in ~11% of the study participants tested. While this failure resulted in a reduction of sample size, the excluded participants did not appear to differ systematically from the study volunteers as a whole (supplemental Table 1). Second, the study was conducted on a highly screened cohort of active duty Marines and Navy corpsmen, which limited the number of participants displaying psychiatric symptoms of sufficient intensity for inclusion in the symptom groups. Therefore, the number of participants included in the symptom groups is relatively small, particularly the depression group. It is possible that low power may have contributed to the inability to detect significant differences in between the depression and healthy control group. However it is important to note that the present findings of normal fear inhibition and extinction in the depression symptom group replicates previous studies with greater subject numbers (Jovanovic et al 2009, 2010). Third, the current study examined effects of psychiatric symptoms in isolation from trauma or deployment history *per se*. By simply comparing LEC scores across symptom groups we found that trauma burden was significantly higher in all symptom groups compared to the healthy group but not different between symptom groups, suggesting that differences in trauma exposure are unlikely to explain differences in task performance across the symptom groups. Future analyses will investigate the role of these variables in influencing task performance, as well as their interaction with psychiatric symptoms. Finally, while the symptom groups had significantly higher scores

on their respective assessment measures relative to the other groups (Table 1), all symptom groups also differed from healthy controls across all measures. This elevation across symptom measures speaks to the difficulty of achieving "pure" symptom categories given the large amount of overlap in phenomenology among these conditions. However, the current paradigm was effective in discriminating between symptom classes based on severity, and as whole it appears that the current results have captured differences between groups characterized by predominant symptoms unique to Anxiety and PTSD. In sum, the fear conditioning and extinction paradigm appears to function as anticipated in this active-duty Marine/Navy cohort, and may, together with the MRS-II study as a whole, lead to novel insights into potential biobehavioral mechanisms of stress injury development, treatment, and prevention.

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Table 1: Demographics and Symptom Measures

	Symptom Group			
	Healthy	PTSD	Anxiety	Depression
N	923	42	37	12
Age	22.23	22.63	22.4	21.38
(SD)	(2.81)	(4.08)	(3.27)	(2.33)
Months in the Military	31.29	39.5	32.7	31
(SD)	(26.18)	(43.89)	(28.74)	(29.64)
Education				
< H.S.	3.3%	2.4%	2.7%	8.3%
H.S.	69.3%	76.2%	73%	91.7%
Some College	25%	21.4%	21.6%	0%
B.A.	2.4%	0%	2.7%	0%
Post-graduate	0%	0%	0%	0%
Rank				
Junior Enlisted	71.3%	76.2%	78.4%	91.7%
NCO	27.5%	23.8%	18.9%	8.3%
Officer	1.2%	0%	2.7%	0%
Race				
White	87.4%	85.7%	83.3%	83.3%
African-American	3.7%	0%	0%	0%
Other	8.9%	14.3%	16.2%	16.6%
Ethnicity				
Not Hispanic or Latino	75.8%	64.3%	67.5%	75%
Hispanic or Latino	24.2%	35.7%	32.4%	25%
Marital Status				
Single, Never Married	68.5%	69%	75.7%	75%
Married	29.3%	28.6%	21.6%	25%
Divorced	1.4%	2.4%	0%	0%
Separated	0.9%	0%	2.7%	0%
Pathology Measures				
(SD)				
CAPS Total Score	9.66[a]	43.74	17.95[a]	27.83[a]

	(9.34)	(11.29)	(10.91)	(12.06)
BAI Total Score	2.87[a]	4.4[a]	20.41	6.67[a]
	(4.03)	(5.54)	(5.45)	(4.92)
BDI-2 Total Score	3.89[a]	9.86[a]	9.65[a]	24.17
	(4.19)	(5.43)	(5.44)	(3.33)
LEC Score	4.16	5.93[b]	5.54[b]	5.92[b]
	(2.80)	(3.60)	(3.12)	(2.27)

[a]=$p<.05$ for comparisons vs category reference group (i.e., PTSD group reference for CAPS score comparisons). [b]=$p<.05$ vs Healthy

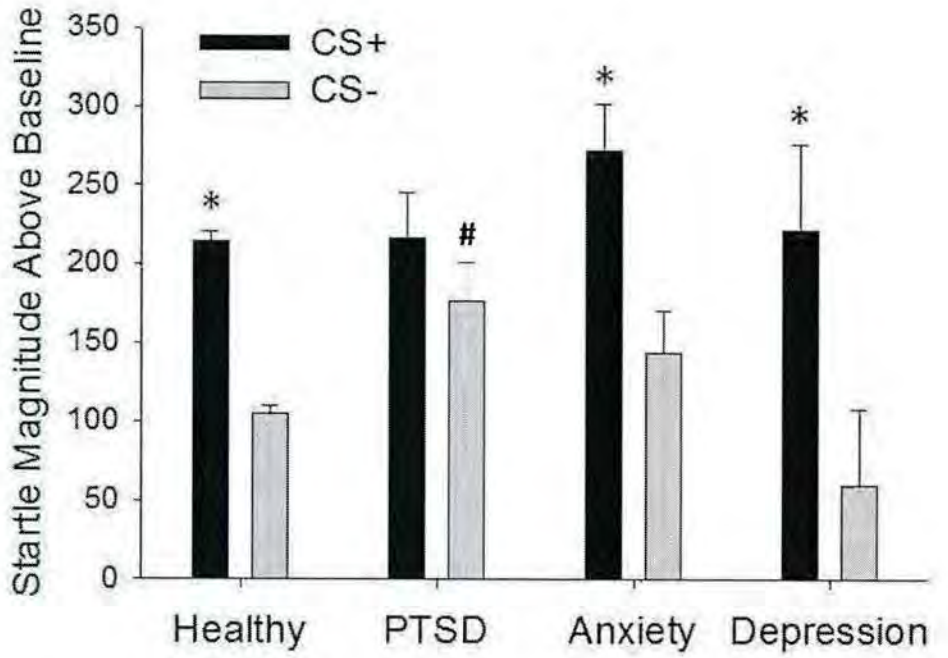
Figure Captions

Figure 1: A) Potentiated startle magnitudes across the last half of the acquisition phase by symptom group. *=p<.05 for CS+ vs CS- comparisons. $^{\#}$=p<.05 for PTSD vs Healthy comparison. B) Expectancy ratings across the last half of the acquisition phase by symptom groups. *=p<.05 for the CS+ vs CS- main effect. C) Self-reported anxiety by symptom groups following the acquisition phase. *=p<.05 for CS+ vs CS- main effect and Anxiety vs Healthy comparison.

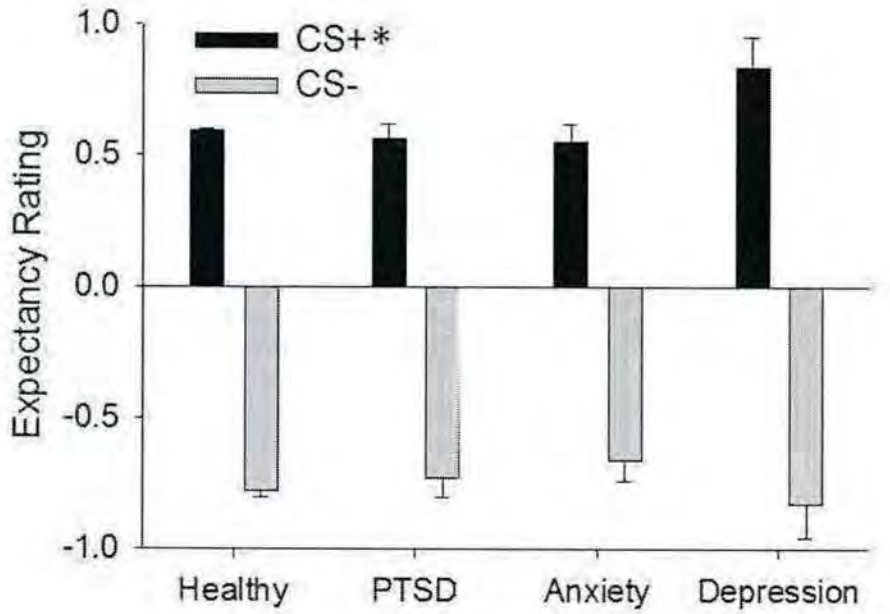
Figure 2: A) % acquisition response retained across the extinction phase by symptom group. *=p<.05 for PTSD vs Healthy comparison. $^{\#}$=p<.05 for exploratory comparisons vs healthy controls. B) CS+ expectancy ratings across the entire extinction phase. C) Self-reported anxiety following the acquisition and extinction phases by symptom group. *=p<.05 for comparisons across phase and for the Anxiety vs Healthy comparison. $^{\#}$=p<.05 for PTSD and Anxiety vs. Healthy comparisons within the extinction phase.

Figure 1: Acquisition

(A)



(B)



(C)

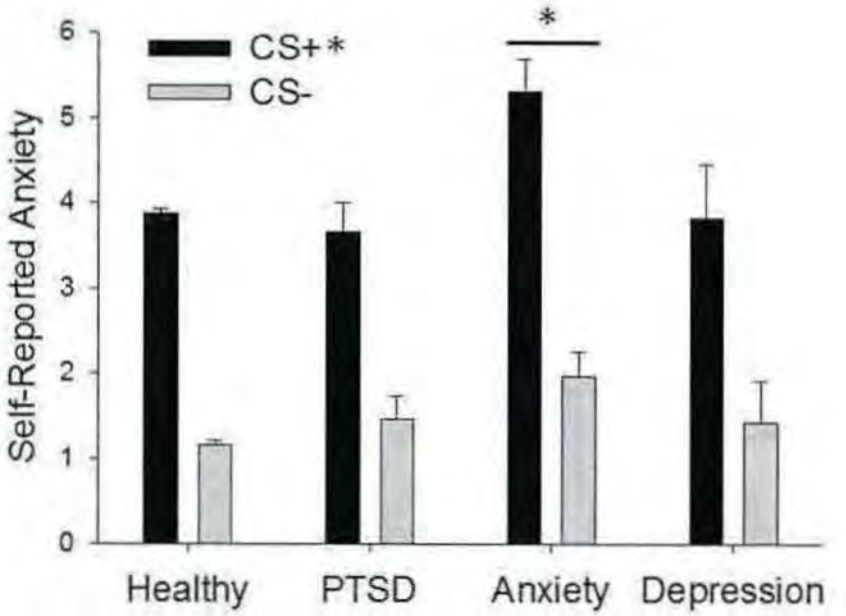
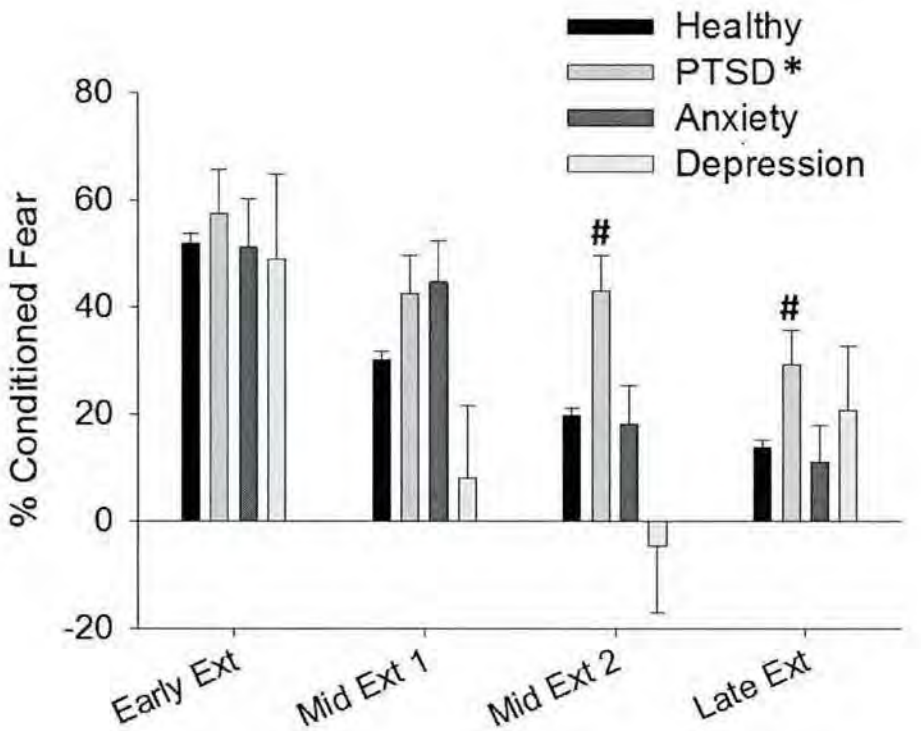
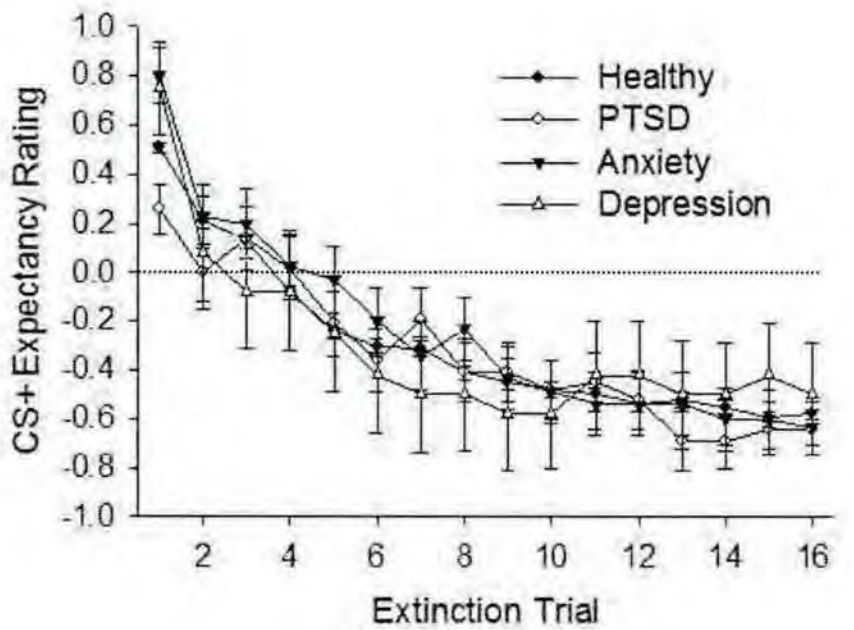

Self-Reported Anxiety
0
1
2
3
4
5
6
CS+*
CS-
*
Healthy
PTSD
Anxiety
Depression

Figure 2: Extinction

(A)



(B)



(C)

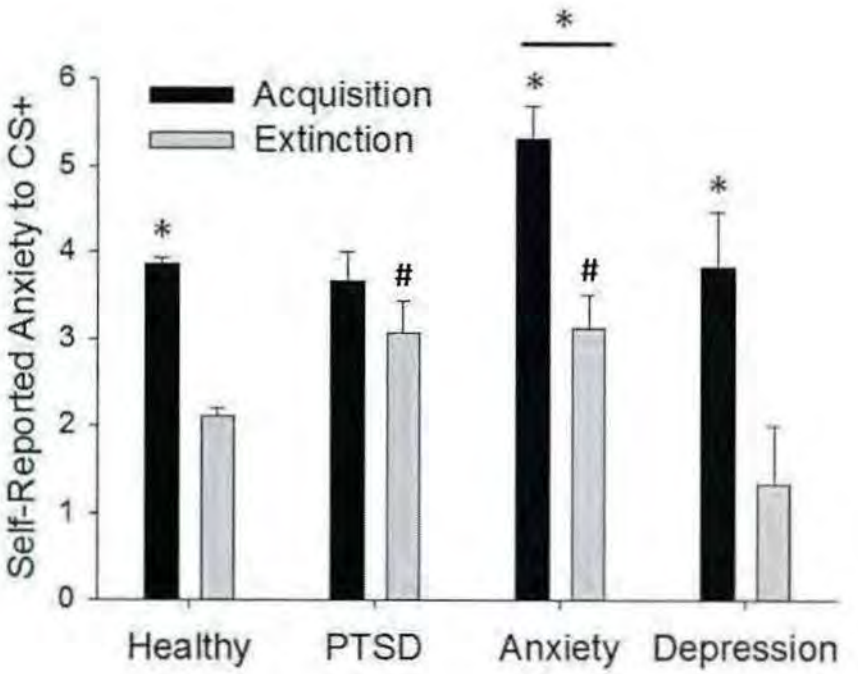
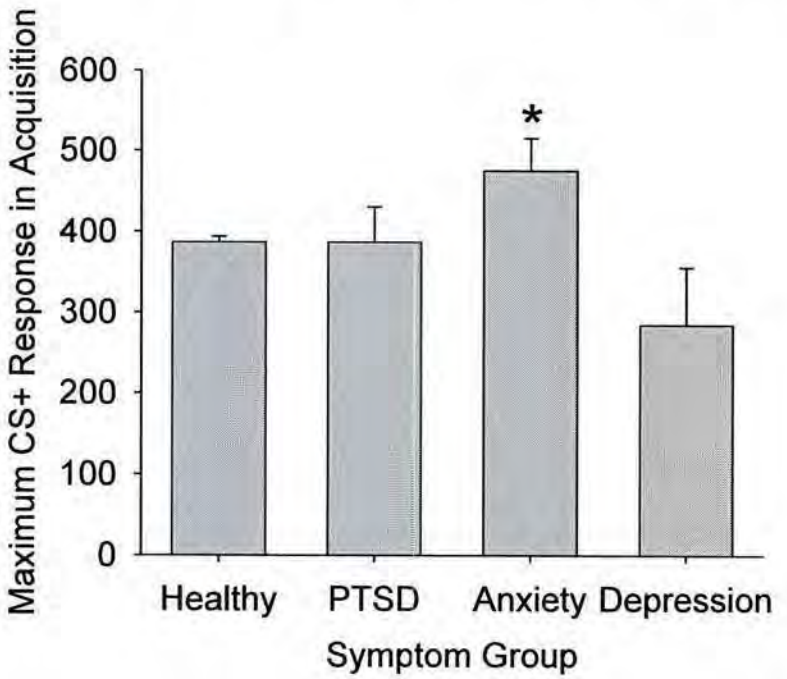

Self-Reported Anxiety to CS+
Acquisition
Extinction
0
1
2
3
4
5
6
*
#
*
#
*
*
Healthy
PTSD
Anxiety
Depression

Table S1. Supplemental Demographics for Subjects Excluded Based on Lack of CS+ Potentiation or Comorbidity

	Lack of Potentiation	Comorbidity
N	125	35
Age	22.28	22.31
(SD)	(3.63)	(3.37)
Months in the Military	30.15	40.26
(SD)	(30.23)	(41.44)
Education		
< H.S.	4%	2.9%
H.S.	72%	71.4%
Some College	21.6%	20%
B.A.	1.6%	2.9%
Post-graduate	0.8%	2.9%
Rank		
Junior Enlisted	77.6%	71.4%
NCO	21.6%	25.7%
Officer	0.8%	2.9%
Race		
White	89.6%	88.6%
African-American	6.4%	5.7%
Other	4%	5.7%
Ethnicity		
Not Hispanic or Latino	74.4%	77.1%
Hispanic or Latino	25.6%	22.9%
Marital Status		
Single, Never Married	76.8%	57.1%
Married	23.2%	42.9%
Divorced	0%	0%
Separated	0%	0%
Pathology Measures (SD)		
CAPS Total Score	12.42	44.43

	(12.64)	(13.42)
BAI Total Score	4.8	20.14
	(6.85)	(8.87)
BDI-2 Total Score	6.01	24.66
	(6.6)	(8.89)
LEC Score	4.26	7.20
	(2.76)	(3.37)

Supplemental Figure 1: Maximal Acquisition Response by Symptom Group.



Conflict of Interest Statement

In the past three years, MAG has received consulting compensation from Abbott, Addex, Cerca, Dart, Lundbeck/Otsuka, Neurocrine, Omeros, Sunovion, Takeda, and Teva, and holds an equity interest in San Diego Instruments. MAG also has research grant support from Intracellular Therapeutics, Johnson & Johnson, NIDA, NIMH, and the U.S. Veteran's Administration VISN 22 Mental Illness Research, Education, and Clinical Center. VBR has received grant funding from Janssen and Omeros. The rest of the authors report no conflicts of interest associated with the current manuscript.

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Drafting of Manuscript: Victoria Risbrough, Dewleen Baker, Dean Acheson, Kate Yurgil, Caroline Nievergelt, Mark Geyer

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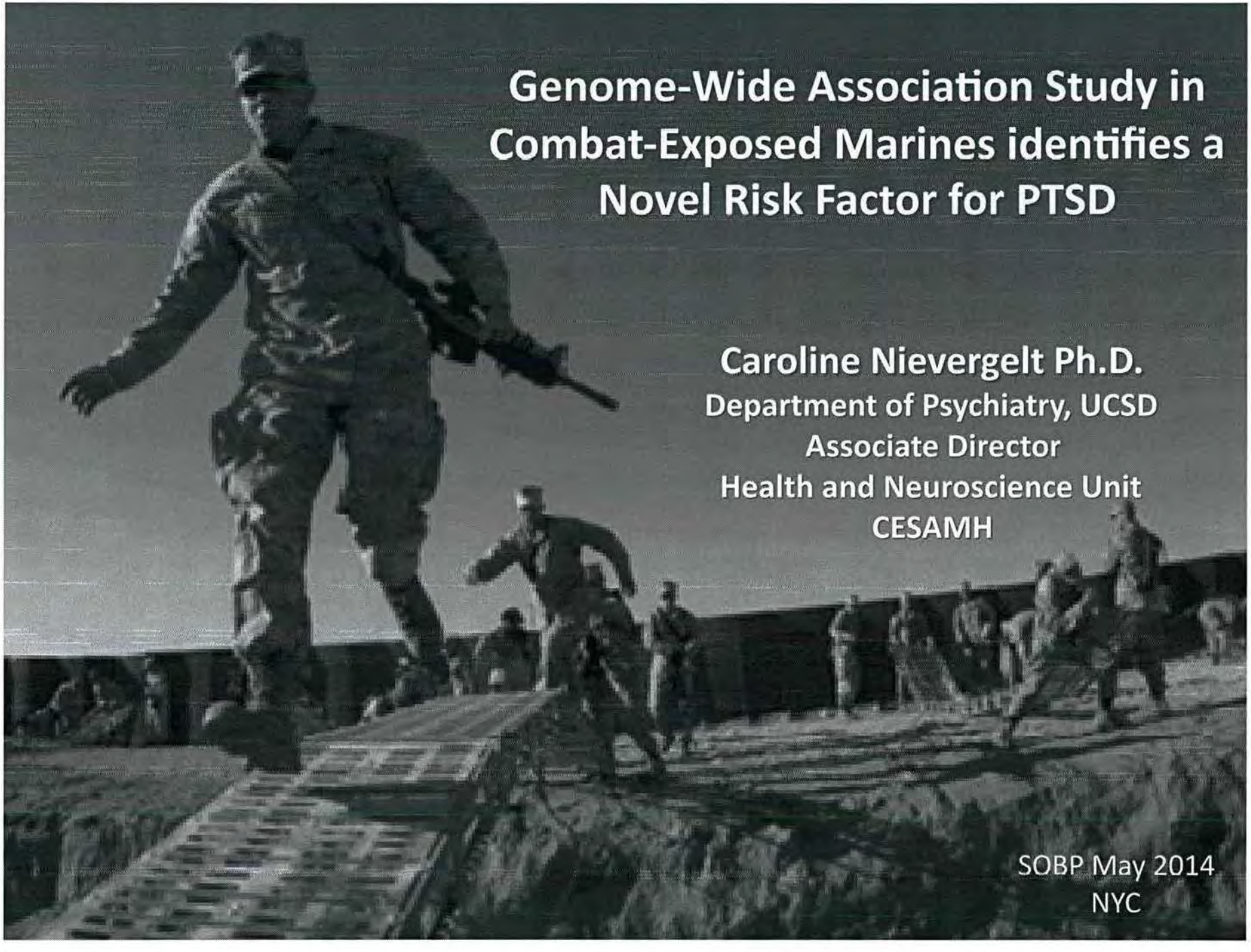
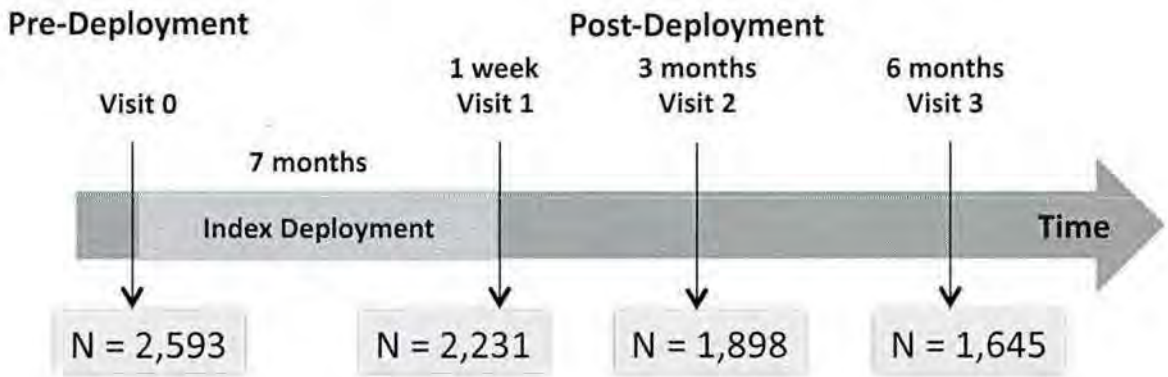

Genome-Wide Association Study in Combat-Exposed Marines identifies a Novel Risk Factor for PTSD
Caroline Nievergelt Ph.D.
Department of Psychiatry, UCSD
Associate Director
Health and Neuroscience Unit
CESAMH
SOBP May 2014
NYC

Marine Resiliency Study (MRS)

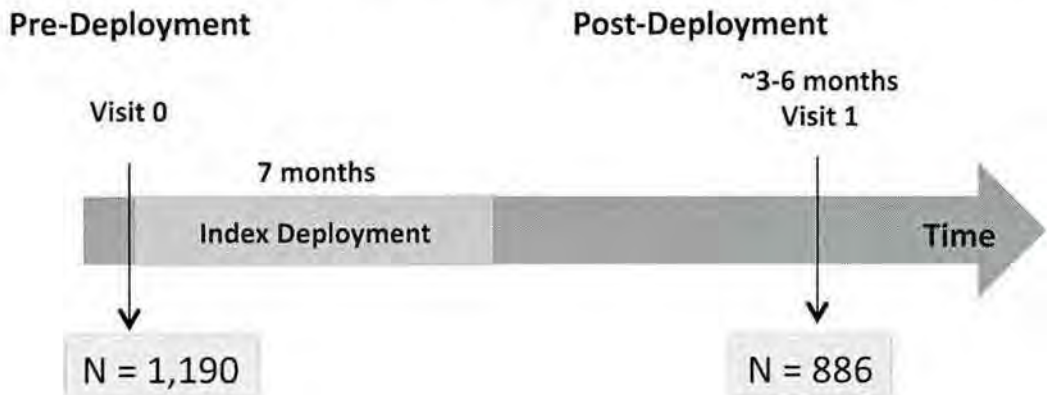
Goal: Predictors of Risk and Resilience for Posttraumatic Stress Disorder

MRS-I: 2008 – 2011 (PIs: Baker, Nash, Litz) Cohorts 1-4



- Prospective study
- Deployment to Iraq or Afghanistan
- Longitudinal follow-up

MRS-II: 2011 – 2013 (PIs: Baker, Risbrough, Geyer) Cohorts 11-12



Timeline and Enrollment

MRS Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews

Historical
- Self-report questionnaires

Neuropsychological
- Attention, Memory, Executive Function, Reasoning, Social Cognition

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

MRS Secure database

Biological assessments

Biomarkers
- NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Metabolomics (subset)

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- Startle threshold and habituation, fear conditioning and extinction, heart rate variability

Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Genomics

- **GWAS (complete data)**
- **Methylome (subset, pre-post)**
- **Transcriptome (subset, pre-post)**

PTSD diagnosis using CAPS

• **Criterion A.** The person has been exposed to a traumatic event in which both of the following were present:
(1) the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others
(2) the person's response involved intense fear, helplessness, or horror (not for military cohorts)

• **Symptom clusters:**
B: Reexperiencing (B1-5)
C: Avoidance / Numbing (C1-7)
D: Hyperarousal (D1-5)

• **F1/I2 rule: frequency of 1 and intensity of 2** **(range: 0-4 for both)**
experienced it at least once or twice during the last month
distress/discomfort: moderate, distress clearly present but still manageable, some disruption of activities

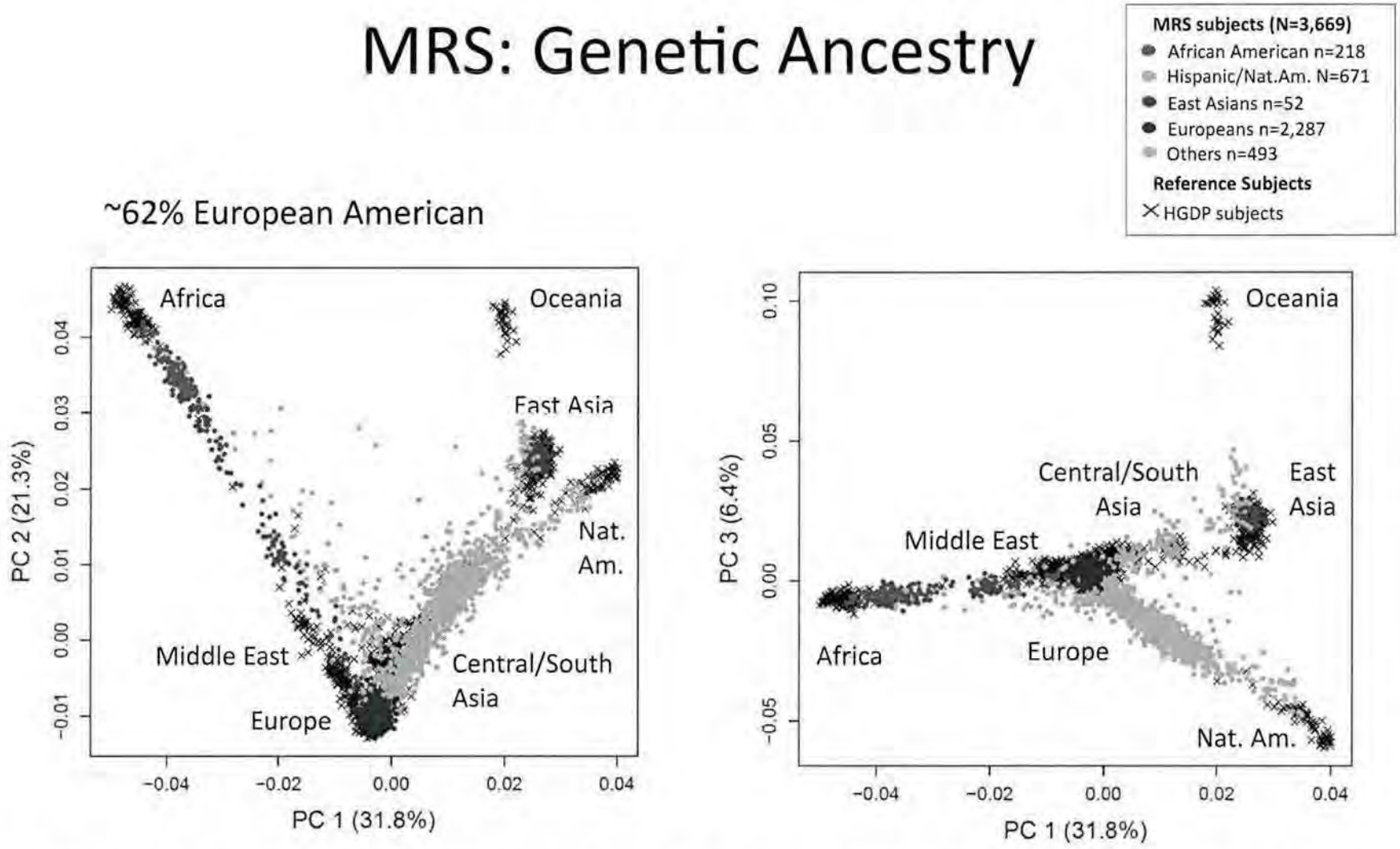
• **DSM-IV PTSD diagnosis:**
Full: 1B, 3C, 2D ← PTSD dx
Partial stringent: 1B, 2C, 2D
Partial lenient: 1B, 3C OR 2D ← Partial PTSD dx

Broad definition of PTSD

• **CAPS summary score:**
Sum of all frequencies and intensities of the 17 questions (range: 0 – 136)

Clinician Administered PTSD Scale (CAPS)

MRS: Genetic Ancestry



- Bayesian based cluster methods (STRUCTURE) to generate ancestry estimates based on HGDP reference populations and AIMs
- Determination of main ancestral groups (<5% admixture)
- Visual inspection: PCA with reference populations and color coding for main ancestral groups

Study design: GWAS on PTSD symptom changes

History:

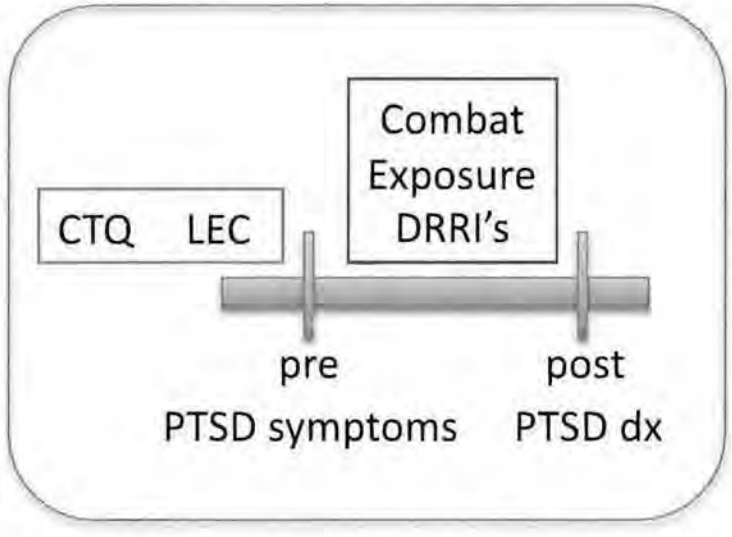
- Psychiatric Genomic Consortium (PGC, 2007): field-wide mega-analyses of genetic associations for ADHD, BPD, MDD, and SCZ
- Most psychiatric disorders are polygenic, with many individual loci conferring only small individual effects
- Very large datasets (>10,000 cases) are necessary for GWAS
- PTSD only recently joined the PGC
- 3 GWAS published to date (*RORA*, *TLL1*, *lincRNA*)
- unique disorder because exposure to a traumatic event is a prerequisite

Rationale:

- Leverage prospective design ⟶ control for PTSD symptoms at predeployment
- Exposed to recent, homogenous trauma (7 month deployment to combat zone)
- Control for individual trauma exposure

Goal: identification of SNPs associated with larger changes in trauma-related symptoms than predicted by the severity of combat trauma exposure

Effect of trauma on PTSD symptoms



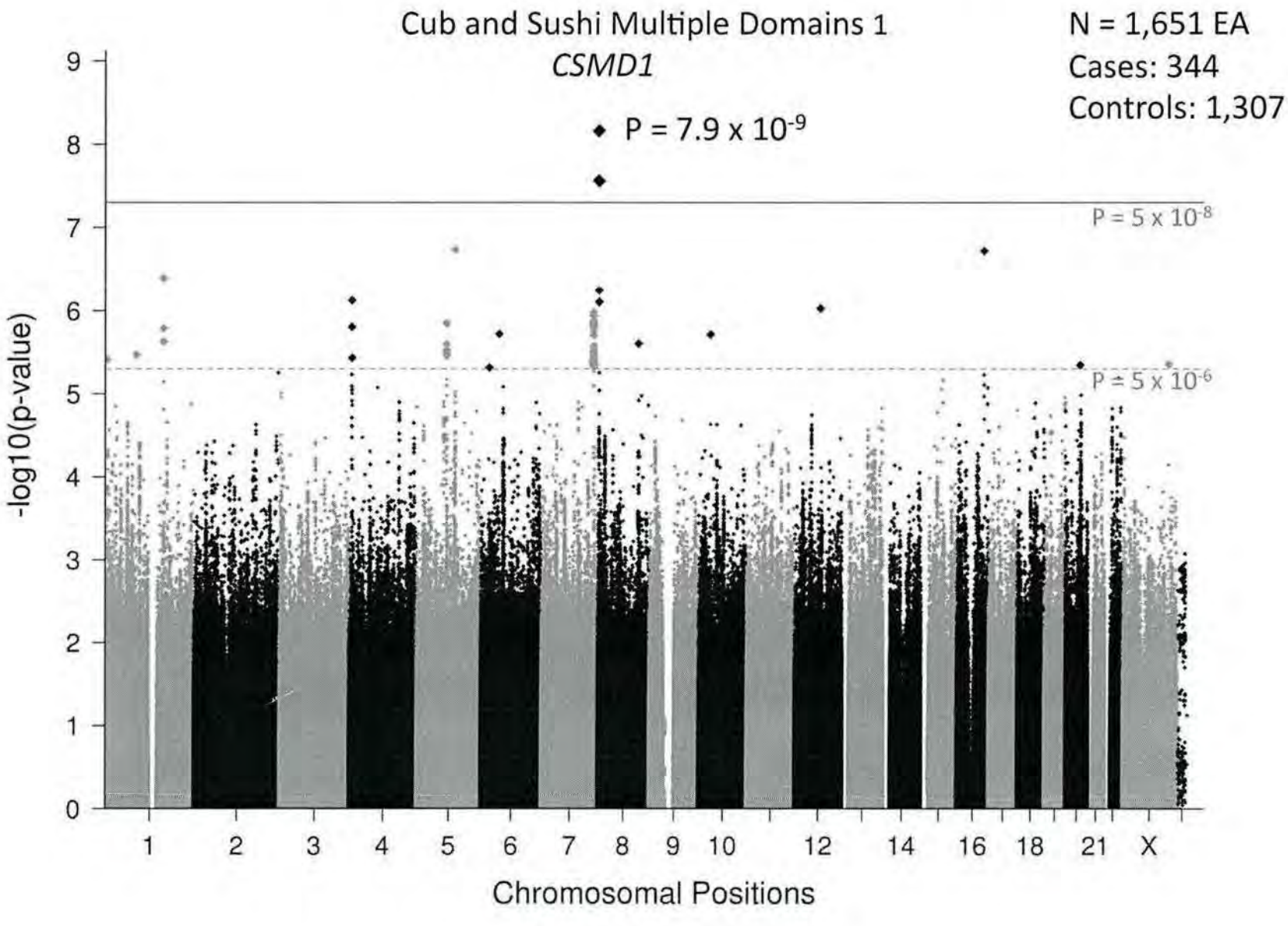
Post-deployment PTSD symptoms as predicted by pre-deployment PTSD and trauma measures:

Variable	% VE	P-value	Cum. % VE	P-cum.
CAPS V0	2.80%	< 2.2e-16	2.80%	< 2.2e-16
DRRI's	2.70%	< 2.2e-16	5.61%	< 2.2e-16
CTQ	1.19%	< 2.2e-16	5.92%	8.92E-10
LEC	0.74%	< 2.2e-16	6.01%	0.0023

DRRI's: composite score of combat exposure measures
CTQ: Childhood Trauma Questionnaire
LEC: Life Events Checklist at pre-deployment (V0)
%VE: % variability explained; cum.: cumulative

Predictors:
- CAPS V0
- DRRI's
- MRS study
- 3 PC's

Post-deployment PTSD case-control GWAS

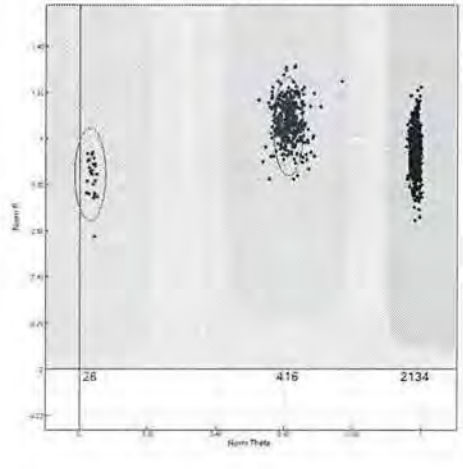


Consistent effects across MRS studies

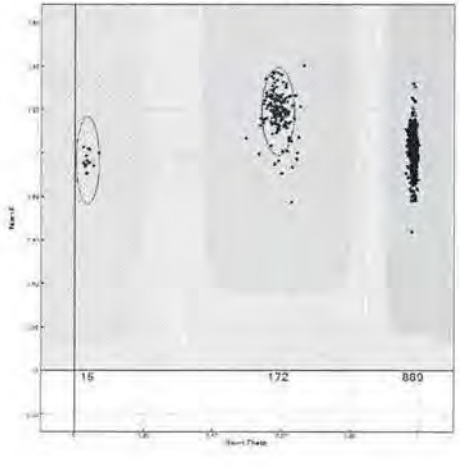
MAF	INFO	OR	SE	P-value	Dataset	N
0.101	0.97	2.27	0.19	**1.49×10^{-05}**	MRS-I	1,129
0.109	1.01	2.38	0.24	**2.77×10^{-04}**	MRS-II	552
0.104	0.98	2.33	0.15	**7.90×10^{-09}**	MRS-I & MRS-II	1,651

Genome studio calls for genotyped SNP

GWAS 1 (Illumina)



GWAS 2 (Rutgers)



Secondary GWAS models

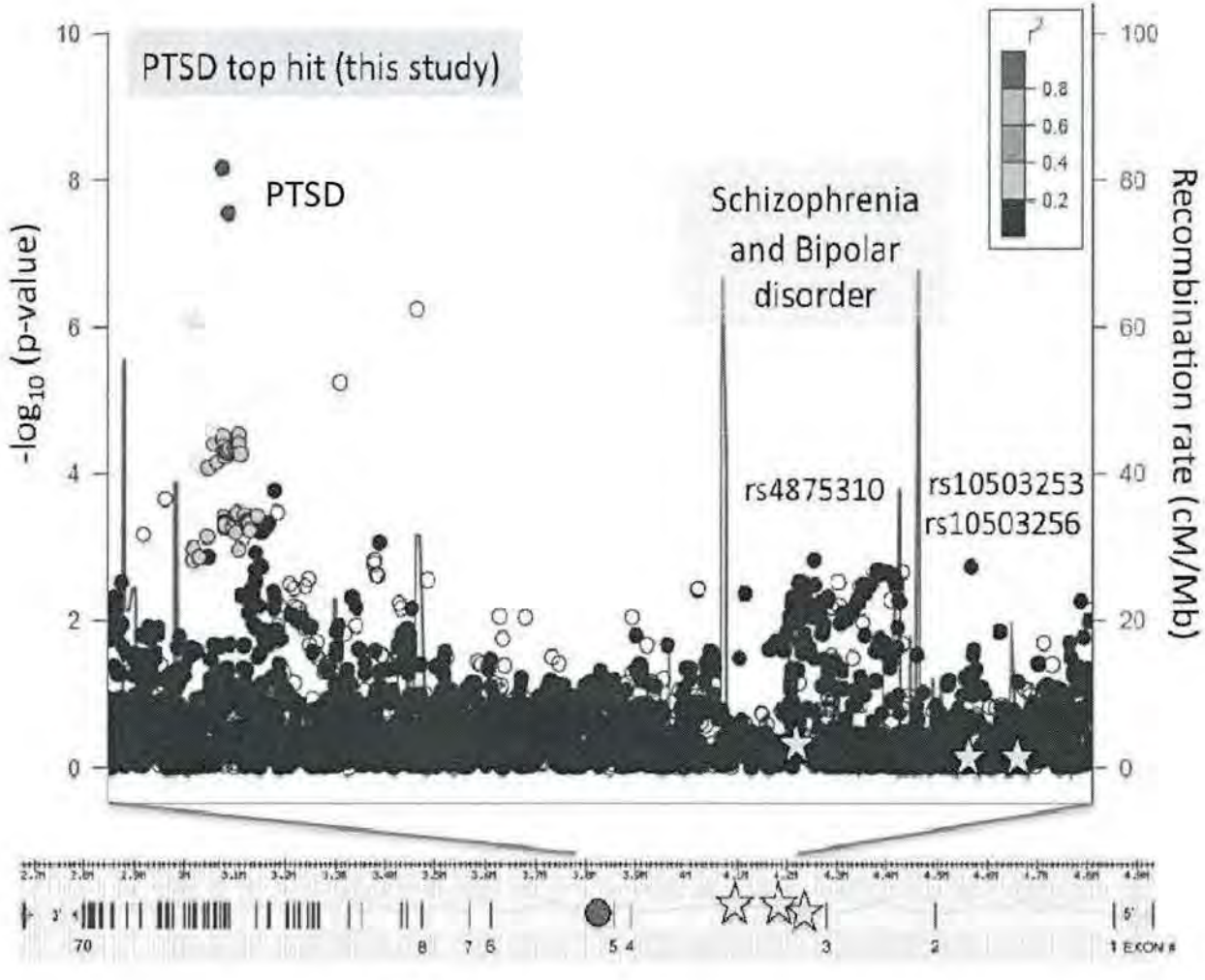
Variable	OR	SE	p	N	AIC	Model
SNP	2.333	0.146	**6.84E-09**	1651	1357.80	base
DRRI's	3.231	0.100	5.40E-32			
caps-qt V0	1.048	0.004	9.42E-26			
SNP	2.372	0.147	**4.66E-09**	1631	**1354.93**	adding CTQ
DRRI's	3.181	0.101	3.54E-30			
caps-qt V0	1.046	0.005	1.55E-22			
CTQ	1.376	0.141	0.024			
SNP	2.398	0.148	**3.27E-09**	1629	1354.95	adding CTQ & LEC
DRRI's	3.177	0.102	6.26E-30			
caps-qt V0	1.044	0.005	1.54E-19			
CTQ	1.326	0.143	0.048			
LEC	1.033	0.023	0.16			

- Adding more trauma variables increases gene effect on PTSD outcome
- Dominant genetic model is more significant than an additive one

CSMD1 function

- not yet fully understood
- gene codes for a multiple domain complement-regulatory protein
- highly expressed in the central nervous system
- In rats:
  - CSMD1 protein blocked classical complement pathway activation
  - primary sites of synthesis are developing CNS and epithelial tissues, suggesting that CSMD1 may be a regulator of complement activation and inflammation in the developing CNS, and may also play a role in growth cone function (Kraus et al. 2006)
- *Csmd1* knockout mice showed behaviors reminiscent of blunted emotional responses, anxiety and depression, suggesting an influence of CSMD1 on psychopathology and endophenotypes of the negative symptom spectra in schizophrenia (Steen et al. 2013)

CUB and Sushi multiple domains-1 gene (*CSMD1*)



Gene size: 2MB

Role of CSMD1 in Neuropsychiatric disorders

- GWAS **schizophrenia locus** (rs10503256) (SCZ Consortium 2011)
- Schizophrenia locus confirmed (rs10503253) (Bergen et al. 2012)
- SNP rs10503256: associated with neurocognitive effects in humans, specifically with poorer performance on neuropsychological measures of general cognitive ability and memory function, **suggesting that CSMD1 may be involved in brain mechanisms related to memory and learning** (Donohoe et al. 2013)
- neural effects of rs10503253 were investigated in vivo in healthy participants in an MRI study, showing reduced cortical activations in the middle occipital gyrus and cuneus, suggesting that CSMD1 may mediate brain function related to **cognitive processes** (Rose et al. 2013)
- GWAS showing supportive evidence for *CSMD1* in **bipolar disorder** (Xu et al. 2014)

MRS Genomic Projects

Subjects: MRS European Americans (N =26 cases and 38 controls, longitudinally)

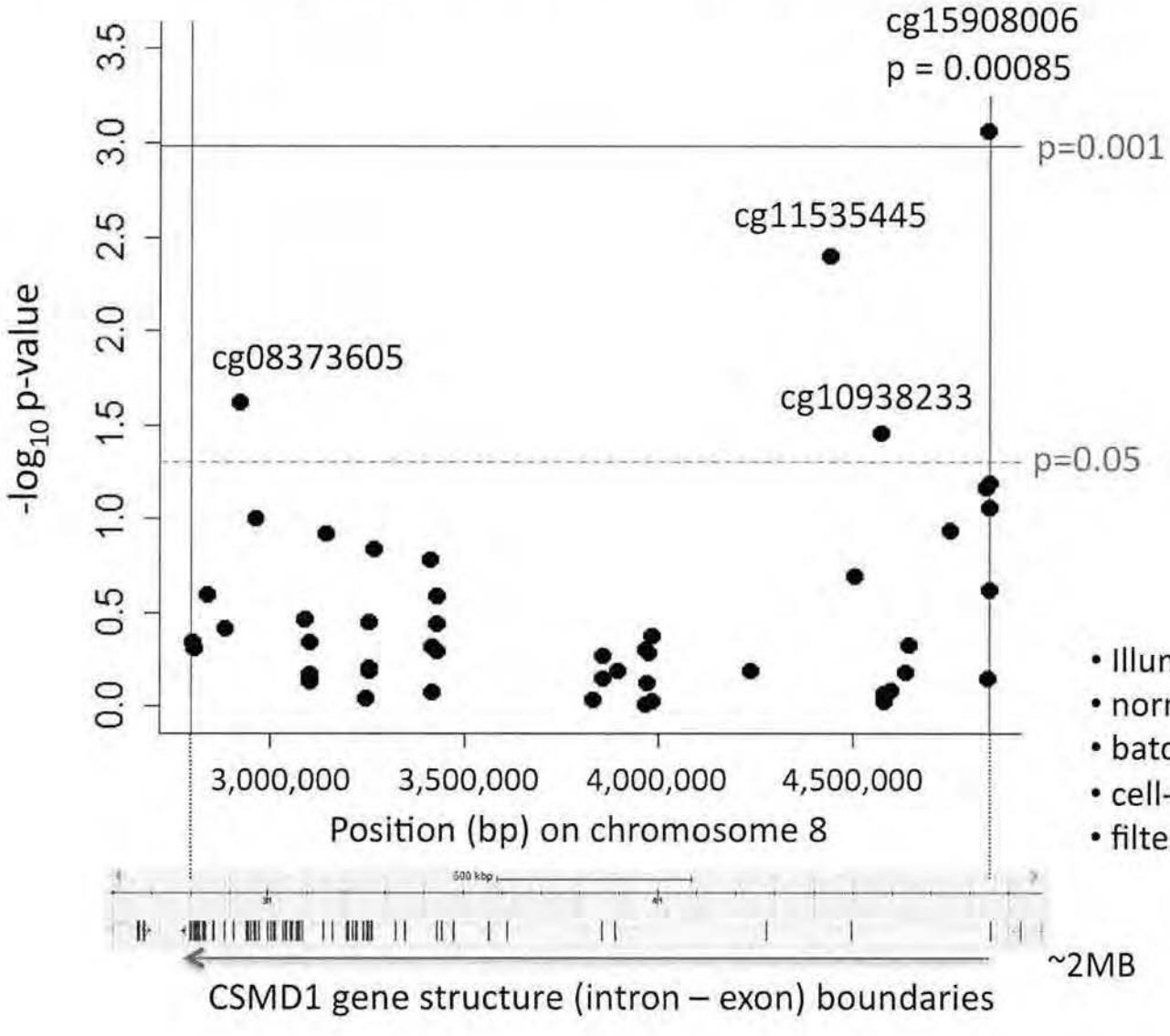
- Transcriptome: Genome-wide gene expression (RNAseq)
- Epigenetic Mechanisms: Genome-wide methylation (Illumina 450K)
- Peripheral blood leukocytes

Demographics of subjects used in the methylation and gene expression analyses

	Controls (n = 38)				Cases* (n = 26)				
Measure	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**	**p**
Assessed pre-deployment:									
Age	22.12	3.47	18.8	34.81	22.6	3.06	19.9	35.05	0.13
CAPs	7.03	7.04	0	21	10.92	6.75	0	22	0.019
Childhood trauma (CTQ)	35.55	15.44	25	103.25	44.25	12.18	26	67.25	0.001
Adult trauma (LEC)	3.45	2.98	0	11	5.27	3.26	1	14	0.011
Tobacco use	1.71	1.94	0	4	2.26	2	0	4	0.30
Assessed post-deployment:									
Combat exposure (DRRIs)	0.61	0.38	0.03	1.49	1.09	0.69	-0.29	2.14	0.002
CAPs	10.45	7.29	0	25	52.19	13.66	15	84	3.8E-11
Tobacco use	1.82	1.61	0	4	1.92	1.76	0	4	0.78

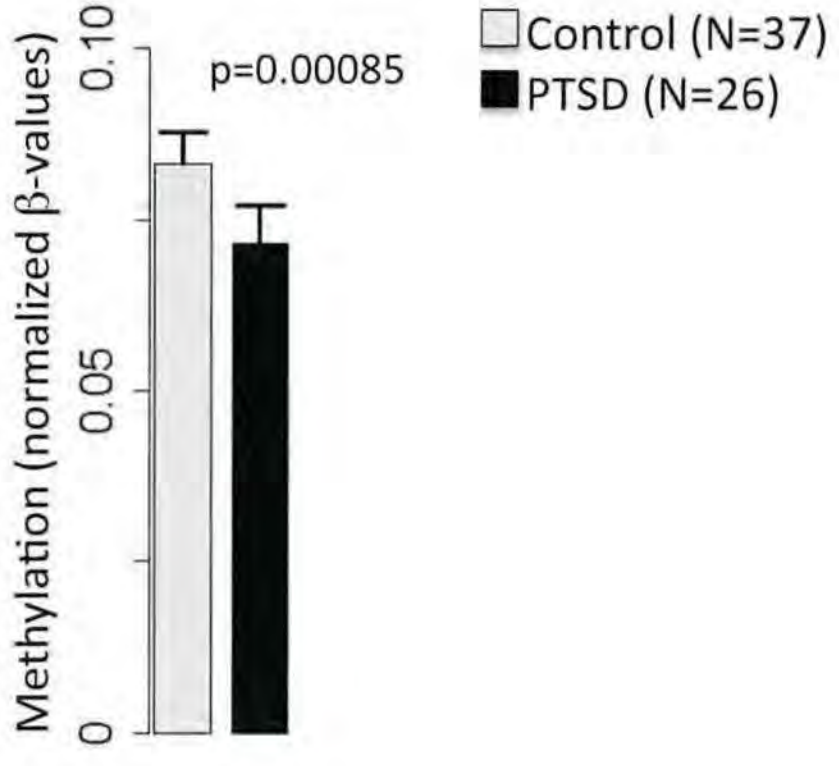
* Cases were selected to be symptom-free (CAPs ≤25) at pre-deployment and diagnosed with partial or full PTSD at the post-deployment visit

Association of PTSD status with post-deployment methylation levels for 48 probes within 50kb of CSMD1 resulting in 4 nominally significant probes



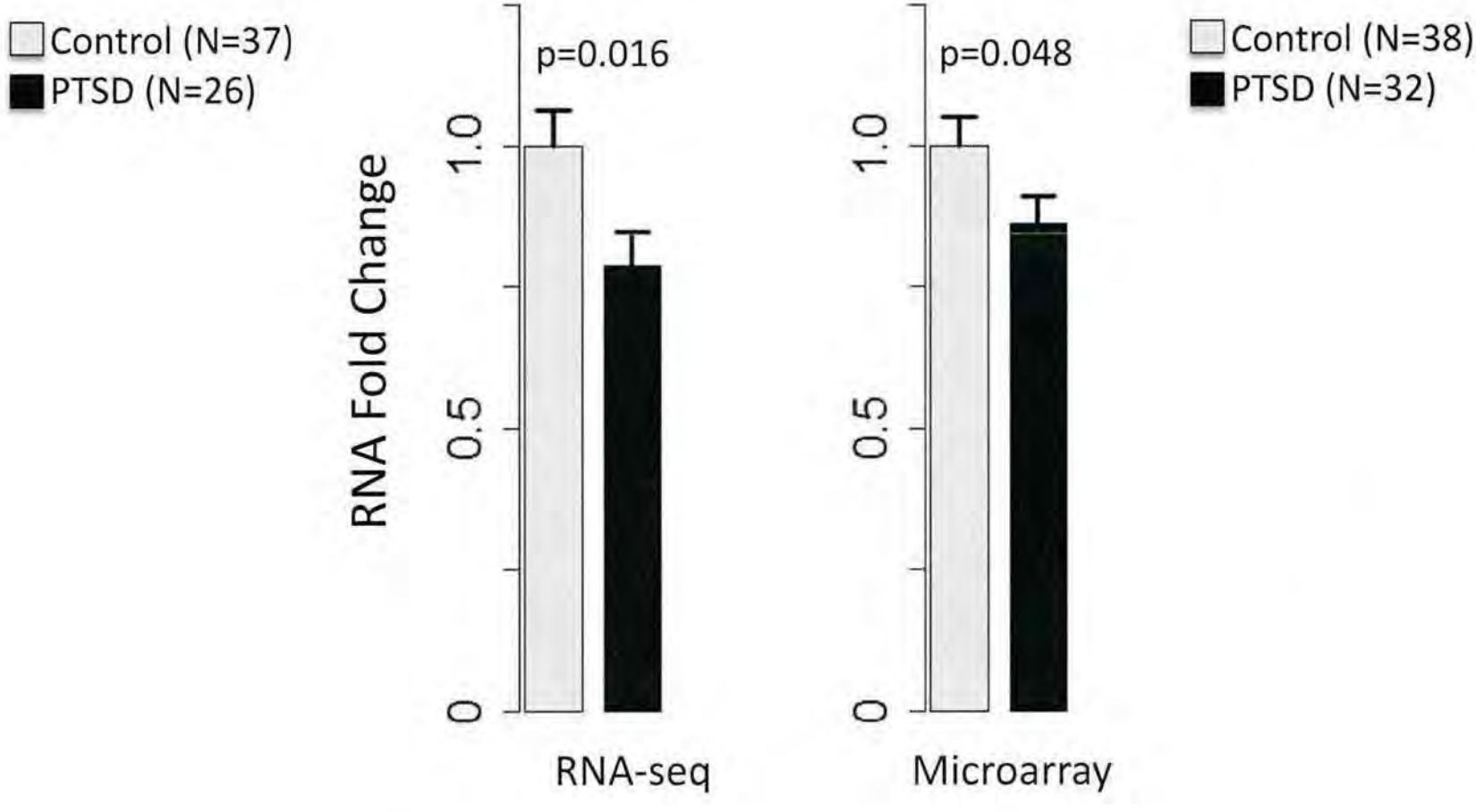
- Illumina 450K array
- normalized β-values (BMIQ)
- batch effects (COMBAT)
- cell-type proportions
- filter on low variability

Mean (±SE) levels of methylation in CSMD1 probe is significantly lower in PTSD cases compared to controls three months after exposure to combat



Linear regression (β-regression) with predictors cases status, predeployment methylation, and 3 PC's

Significantly lower gene expression in CSMD1 in PTSD subjects compared to controls at post-deployment assessments



Data represent fold change ($2^{-\Delta VST}$) ± SE, relative to controls, following normalization and VST transformation

Conclusion

- Genome-wide significant association of *CSMD1* with PTSD
    - awaiting replication in longitudinal cohort
- *CSMD1* association is consistent across MRS-I and MRS-II
- *CSMD1* is associated with SCZ, BPD and neurological traits
- Methylation and gene expression levels are significantly lower in PTSD subjects compared to controls after exposure to combat

Collaborators and Support

Marine Resiliency Study
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NPY CSF and Plasma Concentrations: Associations with Childhood trauma, Combat Stress and PTSD

Dewleen G. Baker, M.D.
Research Director

University of California, San Diego
August 22, 2014

What is Neuropeptide Y?

- Phylogenetically ancient, abundant 36 amino acid peptide
- The most widely expressed peptide in the mammalian brain
- Six known receptor subtypes that couple to G protein complex
- Y1, Y2, Y4, Y5 are functional subtypes in the human brain; high expression in the amygdala
- Activated by NPY, pancreatic peptide and peptide YY
- Y1R tends to serve a "brake" function: is important in regulation of anxiety and fear modulation, tyrosine hydroxylase activity, blood pressure, and bone growth regulation
- Y2R is located pre-synaptically on neurons that contain NPY and negatively regulate release
- Y5R is involved in energy balance, circadian regulation and may be a second brain peptide (in addition to Y1R is important in regulation of anxious temperament (Roosebloom et al. 2014)

Neuropeptide Y

In the brain

- Wide distribution in many brain regions & spinal cord
  Abundant in forebrain limbic structures relevant for behavioral and emotional responses to stressful stimuli/fear/anxiety producing events,
  as well as brainstem (Gray & Morley 1986; Eaton et al 2007 review)
- NPY synthesis by neurons and astrocytes;
  both have NPY receptors (Ramamoorthy & Whim 2008)
- Cross-talk with receptors relevant to psychiatric conditions, e.g. GABA, CRF, NE
- In hypothalamus, NPY participates with leptin and α-MSH in energy balance; in SCN participates in circadian regulation; involved in adult neurogenesis

Neuropeptide Y

In the body

- Co-stored with catecholamine transmitters and co-released released with stimulation of the SNS pathway
- Likely sources of plasma NPY: sympathetic nerve endings thought to be predominant source– also adrenal & platelets

In the periphery

- NPY—in the face of stress— (acutely) amplifies the stress response, can lead to detrimental effects on the body
- Via Y_1 receptor, NPY, with catecholamines, promotes vasoconstriction; *independently* is a long-term regulator
- NPY's angiogenic and immune effects promote atherosclerosis
- NPY's angiogenic effects promote fat cell growth and promote metabolic syndrome

Neuropeptide Y Concentrations: Across the BBB & Across Time

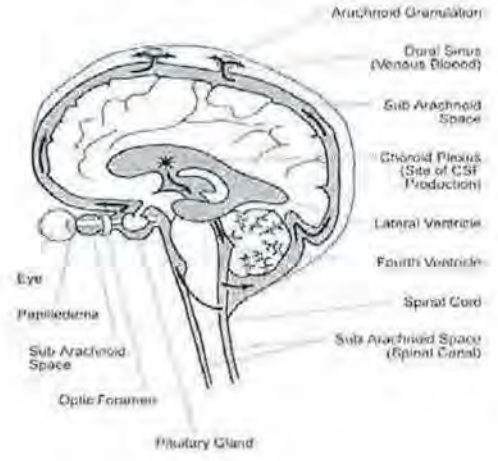
Sources of information:

- Cross-sectional 24-h CSF and Plasma from Serial CSF study
- Longitudinal (pre- and post-deployment) fluids (plasma, urine, saliva) from a large cohort of deployed Marines (MRS study)

Presentation & Discussion

- Presentation of cross-sectional (24-h serial) CSF and plasma NPY concentrations in PTSD, healthy deployed vets & civilians
- To show baseline determinants of plasma NPY levels (predeployment NPY levels)
- To show changes in plasma NPY concentration over time in relation to stress i.e. childhood trauma & combat stress
- Discuss possible implications, mechanisms and future directions

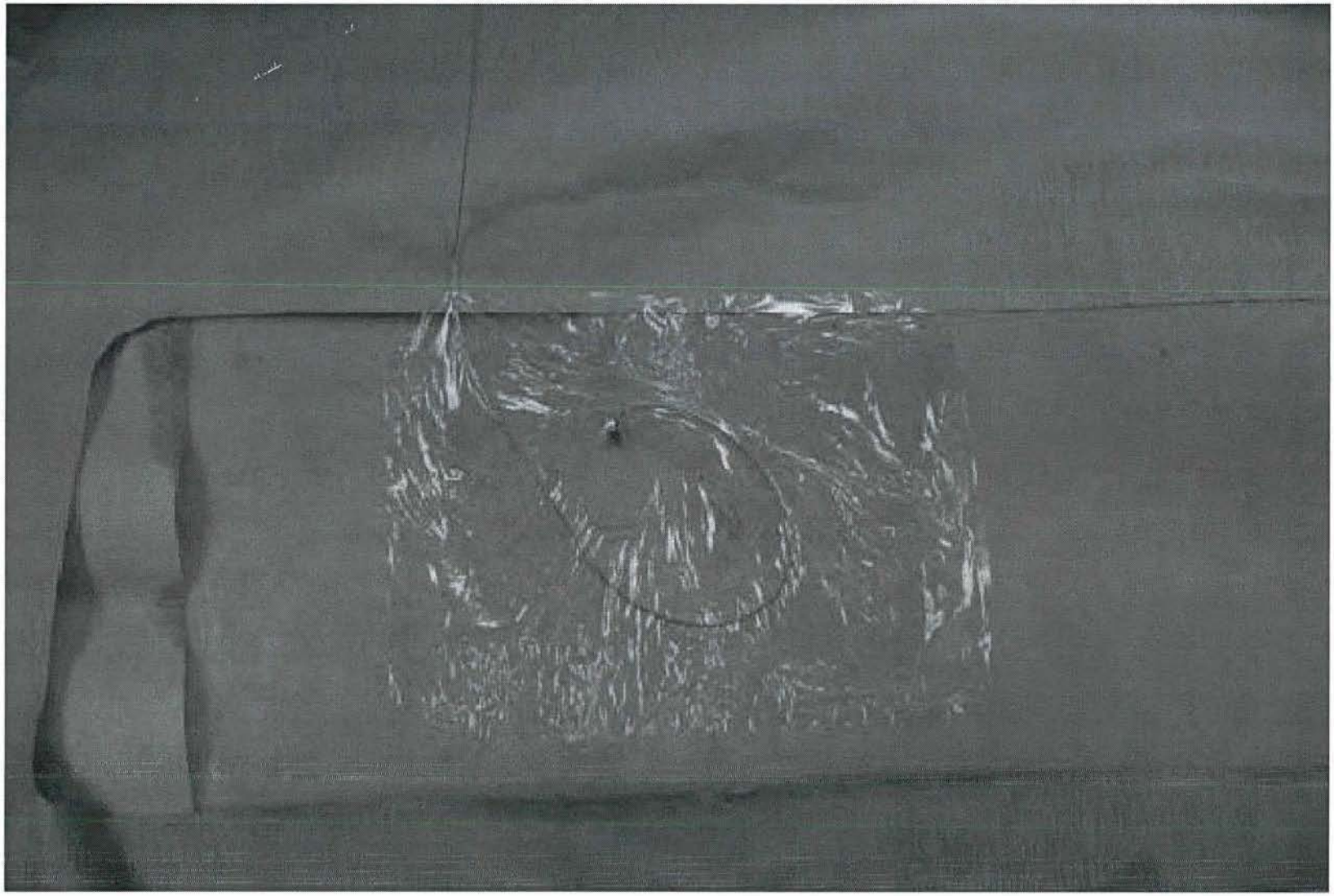
Serial CSF Methodology



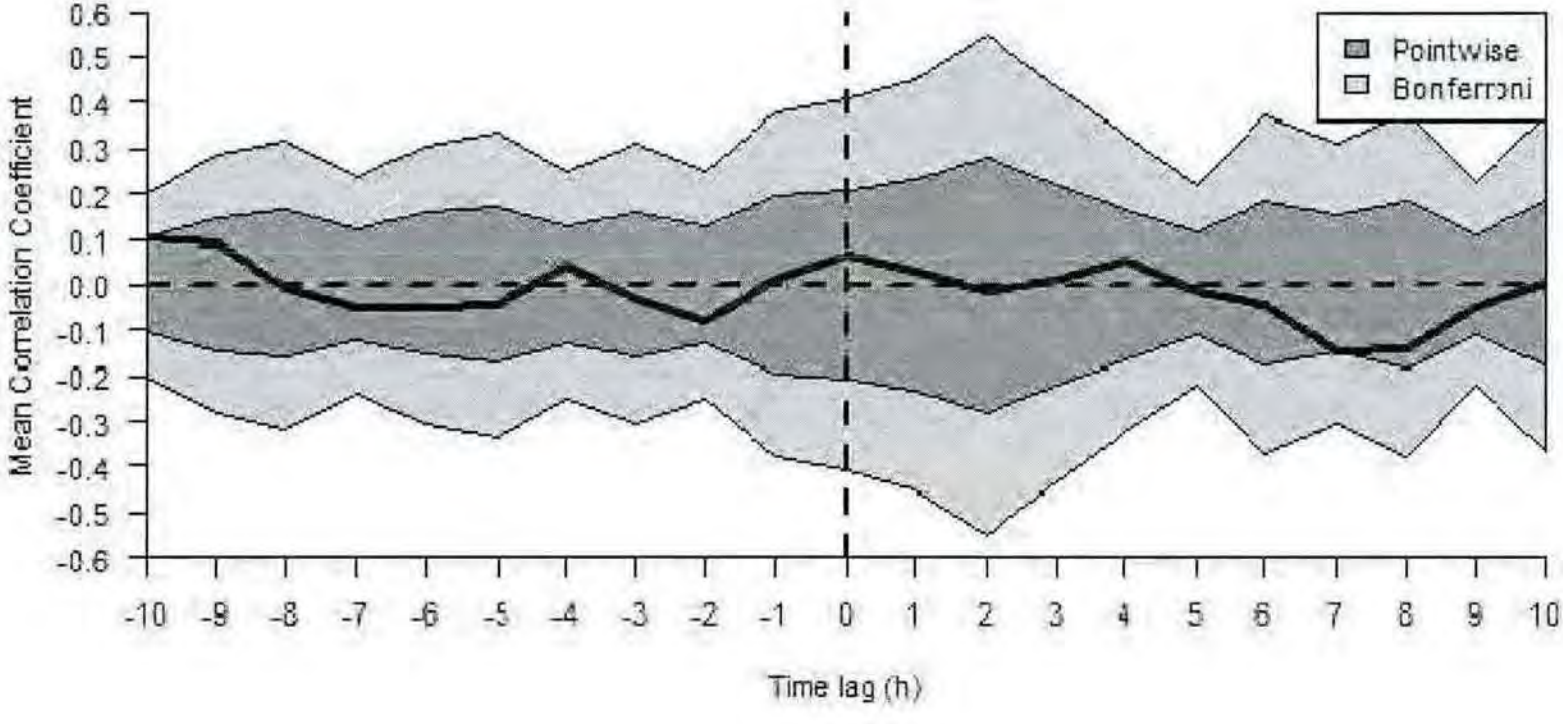
- Serial cerebral spinal fluid study
- Cross sectional study of three groups: PTSD, combat control, non-traumatized civilian volunteers
- Serial CSF, repeated plasma and saliva and 24-h urine collection over a 24 hour timeframe
- Groups well-characterized, matched for age and gender: 26 deployed subjects, veterans and active duty combatants
- of the Iraq and Afghanistan conflicts, 12 subjects with, 14 subjects without PTSD and 12 physically and mentally healthy civilian volunteers
- Eleven subjects with PTSD, 11 civilian volunteers and 14 combat controls had sufficient CSF for statistical analysis

Methodology Catheter Insertion

Methodology: Catheter in place



Cross-lagged correlation: CSF/Plasma NPY

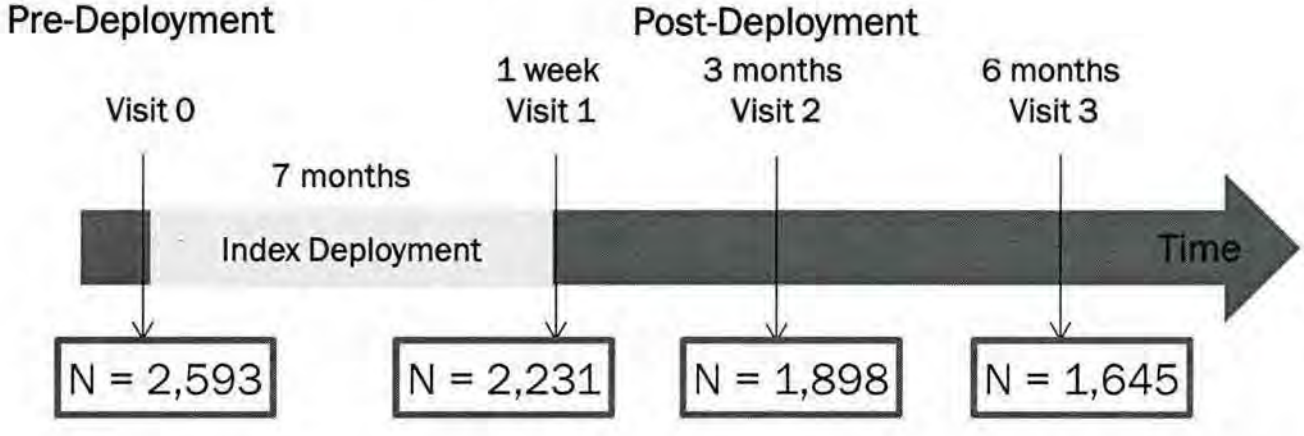


Baker DG et al., PNEC 2013 38(10):2378-82

Marine Resiliency Study (MRS)

Goal: Predictors of Risk and Resilience for PTSD

MRS-I: 2008 – 2011 (Cohorts 1-4)



- Prospective study
- Deployment to Iraq or Afghanistan
- Longitudinal follow-up

Timeline and Enrollment

For Detailed Description of Methods:
See Baker et al. Prev Chronic Dis. 2012;9:E97

MRS Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews

Historical
- Self-report questionnaires

Neuropsychological
- Attention, Memory, Executive Function, Reasoning, Social Cognition

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

MRS Secure database

Biological assessments

Biomarkers
- NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Metabolomics (subset)

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- Startle threshold and habituation, fear conditioning and extinction, heart rate variability

Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Genomics

- **GWAS (complete data)**
- **Methylome (subset, pre-post)**
- **Transcriptome (subset, pre-post)**

PTSD diagnosis using CAPS

• **Criterion A.** The person has been exposed to a traumatic event in which both of the following were present:
•(1) the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others
•(2) the person's response involved intense fear, helplessness, or horror (not for military cohorts)

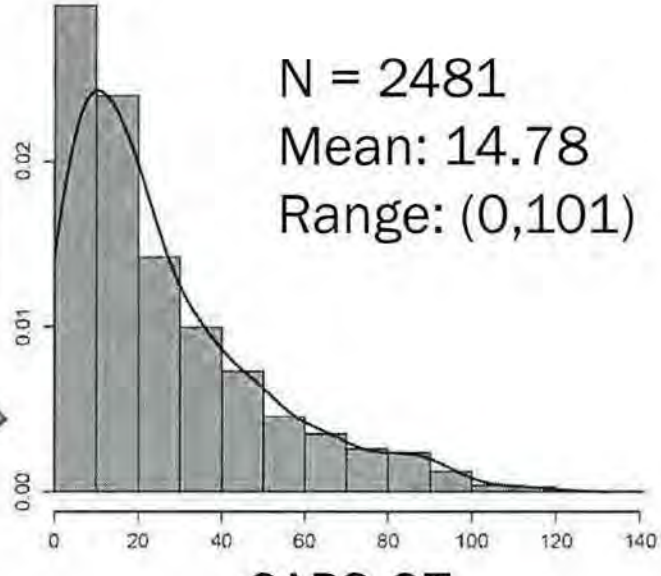
• **Symptom clusters:**
•B: Reexperiencing (B1-5)
•C: Avoidance / Numbing (C1-7)
•D: Hyperarousal (D1-5)

• **F1/I2 rule: frequency of 1 and intensity of 2** **(range: 0-4 for both)**
•experienced it at least once or twice during the last month
•distress/discomfort: moderate, distress clearly present but still manageable, some disruption of activities

• **DSM-IV PTSD diagnosis:**
•Full: 1B, 3C, 2D ← PTSD dx
•Partial Stringent: 1B, 2C, 2D ← Partial PTSD dx

• **CAPS summary score:**
• Sum of all frequencies and intensities of the 17 questions (range: 0 – 136)

• **Zero-Inflated Binomial Model (ZINB)**
Zero-inflated negative binomial regression is for modeling count variables with excessive zeros and it is usually for overdispersed count outcome variables.



Clinician Administered PTSD Scale (CAPS)

Sociodemographic and psychometric data at baseline (n = 2489)

Psychometric data			
BDI Total Score	6.7 (0-51)	CTQ Total Score	40.19 (25-106.5)
Depression°	198 (7.7%)	Tobacco Use	1401 (56.3%)
CAPS summary score	14.8 (0-101)	AUDIT total score	9.6 (0-37)
PTSD Diagnosis[a]	176 (7.1%)	Alcohol Abuse*	326 (15.8%)
Demographic Information			
Race		Age (yrs)	22.8 (18–47)
Black/African American	115 (4.6%)		
Caucasian	2068 (83.1%)		
Other	306 (12.3%)		

° ≥ 20 Beck Depression Scale

[a] DSM IV Full and partial stringent DX

* Alcohol abuse = audit score > 2

Childhood trauma data at baseline (n = 2489)

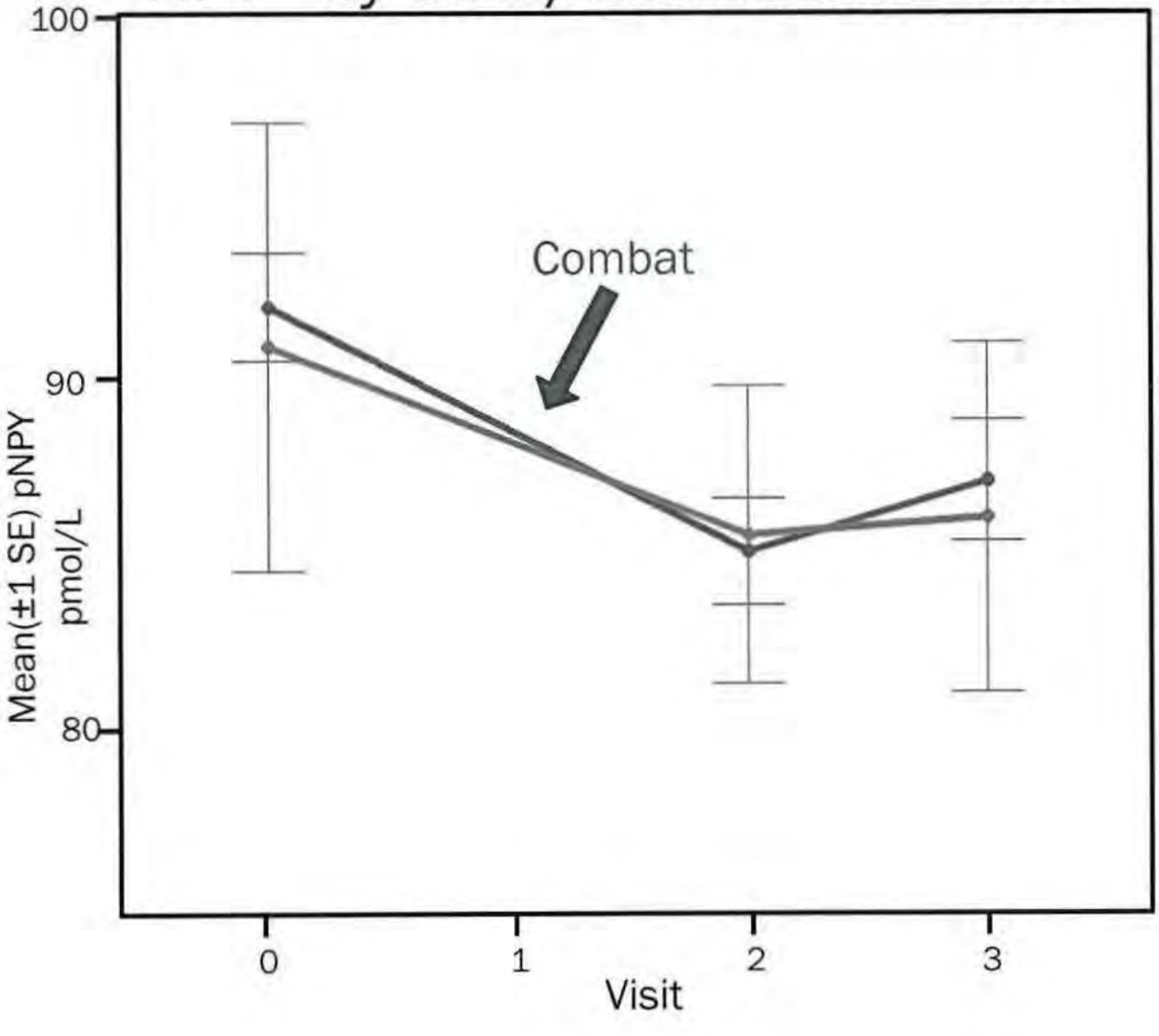
Childhood trauma prevalence			
Childhood Trauma		**CT Type (% with)**	
No CT	1320 (53.4%)	Emotional Abuse	183 (11.9%)
One CT	538 (21.8%)	Physical Abuse	516 (33.5%)
Multiple CT	614 (24.8%)	Sexual Abuse	110 (7.2%)
2 CT	287 (11.6%)	Emotional Neglect	300 (19.5%)
3 CT	162 (6.6%)	Physical Neglect	366 (23.8%)
4 CT	134 (5.4%)		
5 CT	31 (1.3%)		

Determinants of Plasma NPY Concentration Prior to and after exposure to combat: A longitudinal assessment

- Evidence for an effect of genotype (rs16147) on plasma NPY concentrations as well as on amygdalar signal (Zhou et al. 2008), & of an interaction between childhood maltreatment & amygdalar signal (Opmeer et al. 2014)

- Evidence from a cross-sectional study that trauma exposure, but not PTSD associated with plasma NPY levels (Morgan et al. 2003) & that high levels of stress during childhood associated with reduced plasma NPY (Jiminez-Vasquez et al., 2001; Husman et al., 2002).

MRS: Mean Levels of Plasma NPY* by Case/Control over time



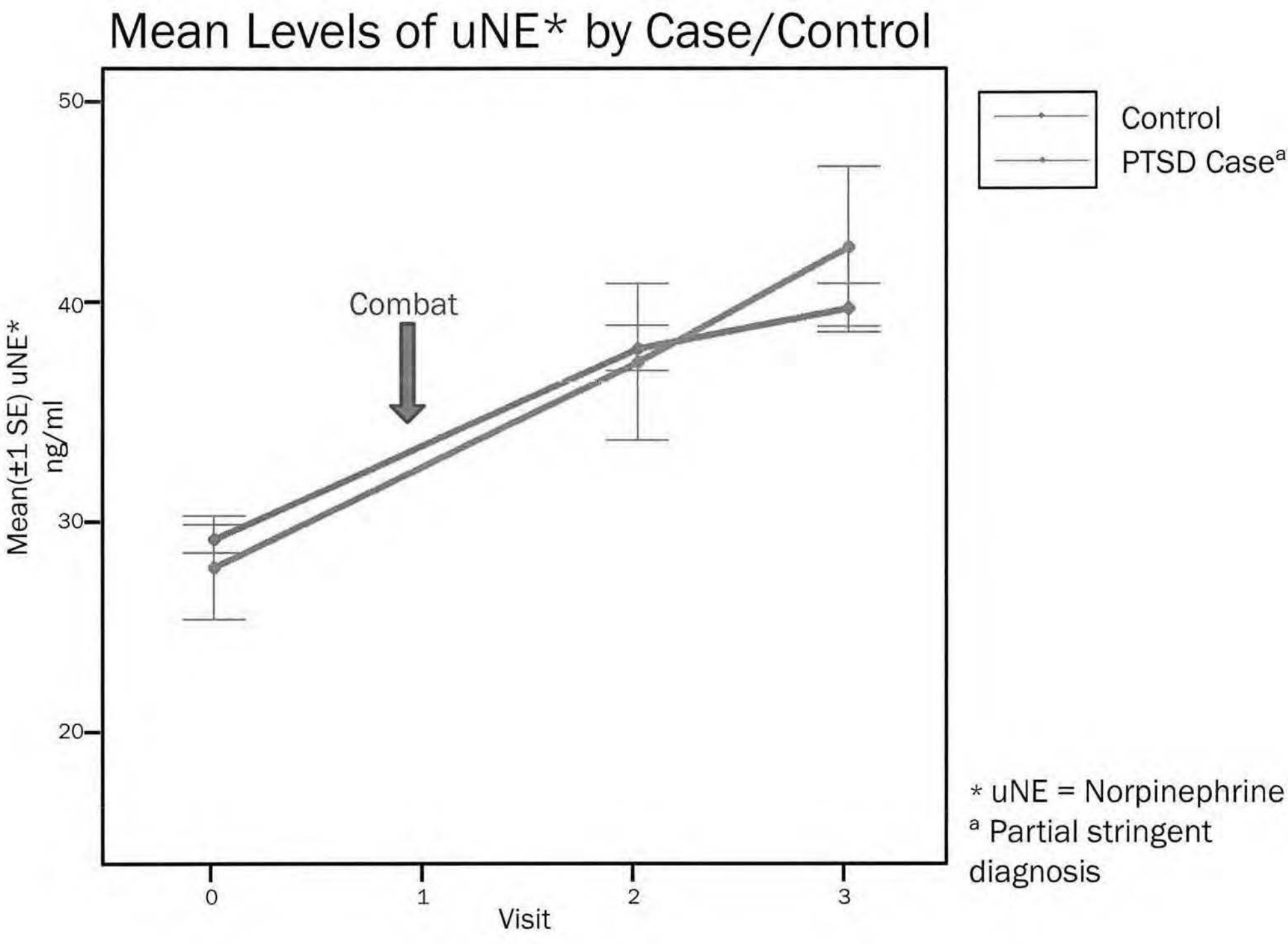
Control

PTSD Case[a]

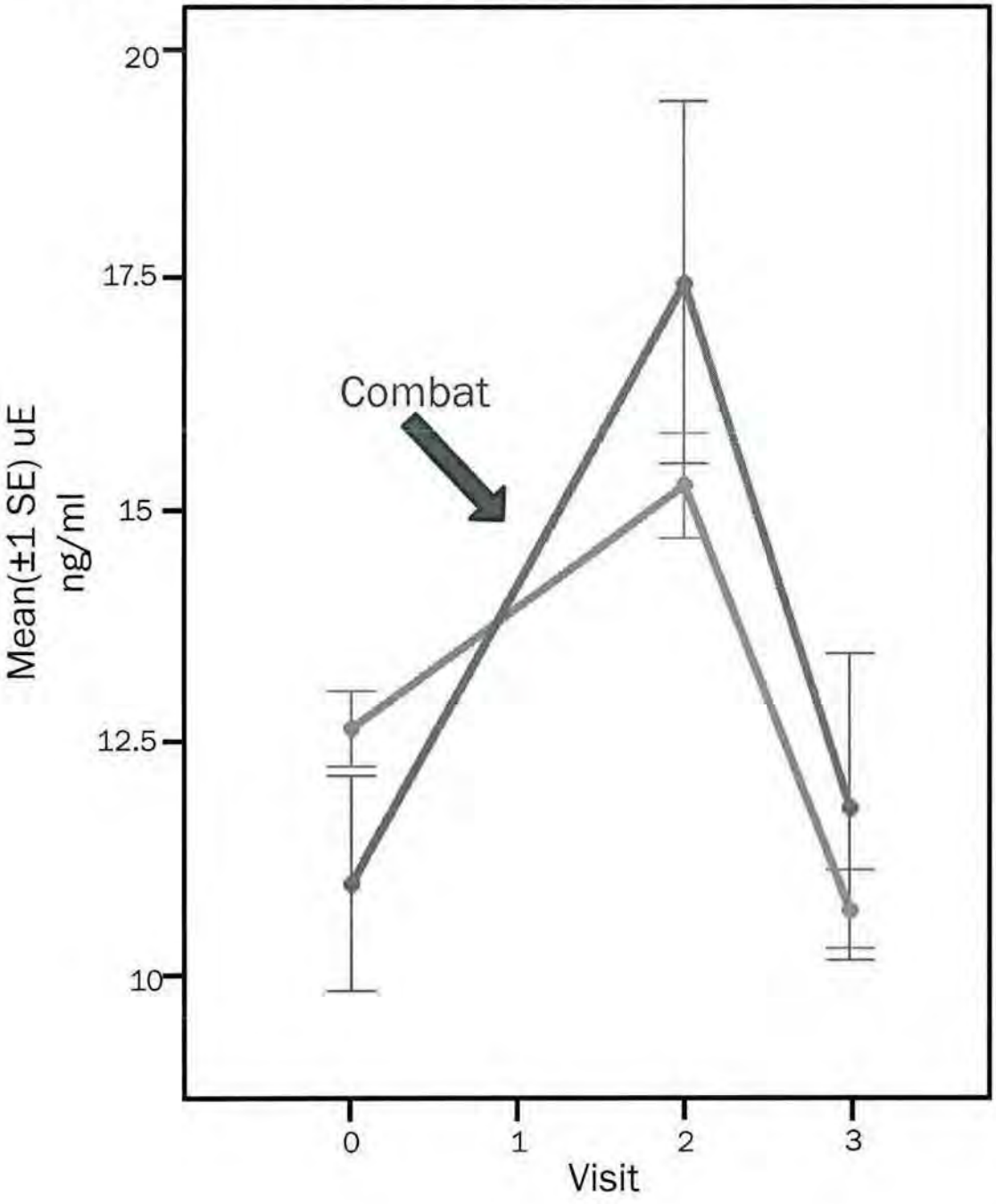
- 3 month post-deployment levels of pNPY drop from baseline levels
- 6 month post-deployment pNPY levels do not recover to pre-deployment levels

* Neuropeptide Y

[a] Partial stringent diagnosis


Mean Levels of uNE* by Case/Control
50
40
30
20
Mean(±1 SE) uNE*
ng/ml
Combat
0
1
2
3
Visit
Control
PTSD Case[a]
* uNE = Norpinephrine
[a] Partial stringent diagnosis

Mean Levels of uE* by Case/Control



* uE = Epinephrine

[a] Partial stringent diagnosis

Correlations between pNPY and Stress Biomarkers

Correlations with pNPY (p-value)			
	Visit 0	Visit 2	Visit 3
uE	-0.02 (0.2)	-0.006 (0.8)	-0.04 (0.1)
uNE	0.02 (0.2)	-0.008 (0.7)	0.06 (0.2)
sCortisol	0.05 (0.1)	0.02 (0.6)	0.002 (1.0)

Correlations between Plasma NPY, spot urine uE, uNE and saliva Cortisol:
Non-significant at baseline (predeployment); non-significant at 3 and 6 months post-deployment

Published in final edited form as:
J Am Coll Cardiol. 2012 October 23; 60(17): 1678–1689. doi:10.1016/j.jacc.2012.06.042.

Neuropeptide Y (NPY):

Genetic Variation in the Human Promoter Alters Glucocorticoid Signaling, Yielding Increased NPY Secretion and Stress Responses

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Abstract

Objectives—This study sought to understand whether genetic variation at the Neuropeptide Y (*NPY*) locus governs secretion and stress responses in vivo as well as *NPY* gene expression in

NIH-PA Author Manuscript

- Studied healthy twin/sibling pairs (n = 399 individuals), typing 6 polymorphisms spanning the locus; replication in 361 MRS Marines & 2212 Australian twins
- Basal/resting plasma concentration of NPY is under substantial genetic control, with heritability (h^2) 0.73 0.04 (p=3.1E-26)
- Haplotype and single nucleotide polymorphism analyses indicated that proximal promoter variant ∇−880Δ (2-bp TG/—, Ins/Del, rs3037354) minor/Δ allele disrupts glucocorticoid signaling to influence NPY transcription and secretion associated with several heritable (h^2) stress traits: NPY secretion (h^2 = 73 ± 4%) as well as greater BP response to environmental (cold) stress, and higher basal systemic vascular resistance.
- rs3037354 used for statistical models in this talk

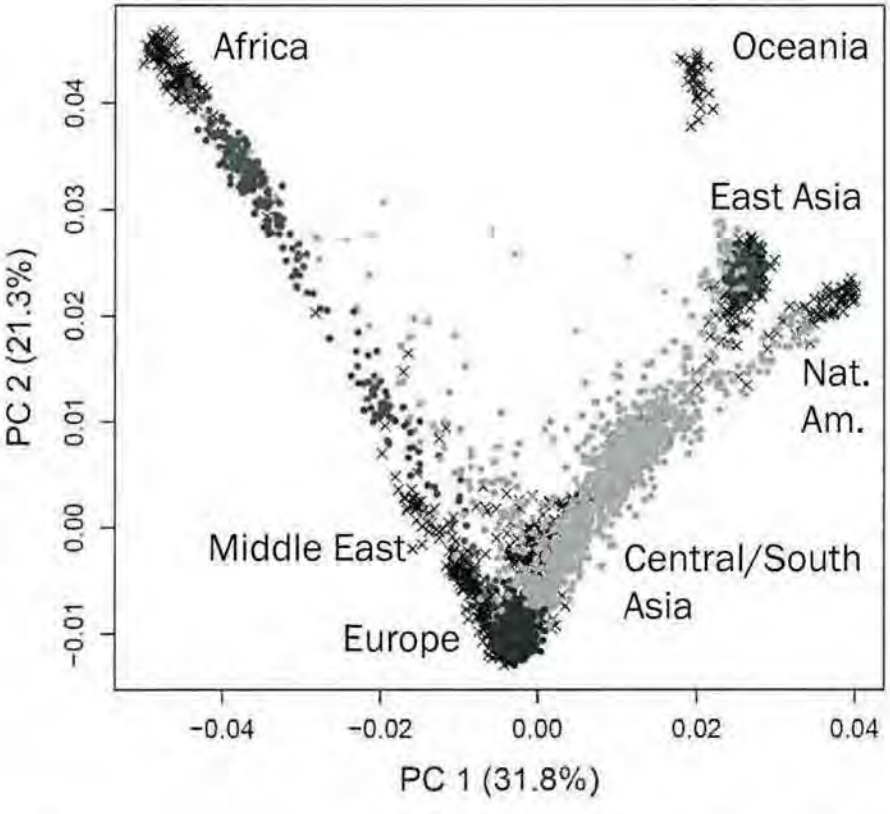
Zhang, et al., 2012, JACC

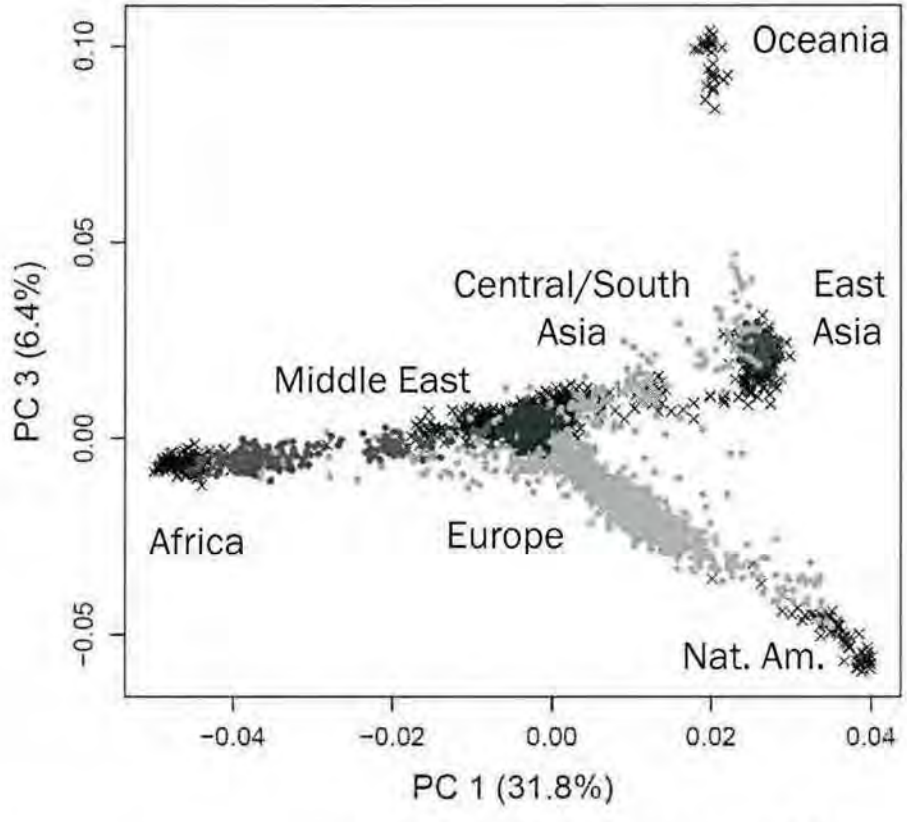
MRS GWAS: Genetic Ancestry

MRS subjects (N=3,669)
- African American n=218
- Hispanic/Nat.Am. N=671
- East Asians n=52
- Europeans n=2,287
- Others n=493

Reference Subjects
- × HGDP subjects

~62% European American





- Bayesian based cluster methods (STRUCTURE) to generate ancestry estimates based on HGDP reference populations and AIMs
- Determination of main ancestral groups (<5% admixture)
- Visual inspection: PCA with reference populations and color coding for main ancestral groups

Nievergelt et al., 2014

Predictors of pre-deployment levels of pNPY

Variable	Beta	SE	t value	p value
Intercept	9.0	0.3	31.8	2.9e-184
Age	0.02	0.01	1.3	0.2
rs3037354	0.3	0.07	4.2	2.6e-05
Prior Deployment	-0.2	0.09	-2.7	0.008
Childhood Trauma	-0.2	0.09	-2.0	0.047

- N = 2276 Marines
- Childhood Trauma Questionnaire (CTQ)
  Scored as 0-1 trauma vs 2 or more traumas (Agorastos et al., 2014)
- rs3037354 Δ-880▽, promoter polymorphism*
- Adjusted for baseline pNPY levels, ancestry, and prior deployment status

Linear Regression

GxE: There is no significant interaction between childhood trauma and rs3037354

Variable	Beta	SE	t value	p value
Intercept	9.1	0.3	31.8	2.5e-184
Age	0.02	0.01	1.3	0.2
rs3037354	0.2	0.08	3.2	0.001
Prior Deployment	-0.2	0.09	-2.7	0.008
Childhood Trauma	-0.2	0.1	-2.1	0.04
Childhood Trauma* rs3037354	0.1	0.1	0.8	0.4

- N = 2276 Marines
- Adjusted for ancestry and prior deployment status.

Linear Regression

Intensity of Combat Exposure is a predictor of post-deployment pNPY levels

Variable	Beta	SE	t value	p value
Intercept	6.3	0.3	20.9	7.2e-85
Age	-0.01	0.01	-0.9	0.4
Baseline pNPY	0.3	0.02	16.2	2.9e-52
Combat Exposure	-0.2	0.05	-3.4	0.0007

- N=1756 Marines in 4 battalions
- Combat exposure: Composite measure of 4 exposure scales (DRRI Combat Experiences, Post-Battle Experiences, Deployment Concerns, Deployment Environment)
- Analysis for adjusted for baseline pNPY levels and ancestry (NS)

Linear Regression

Model including childhood trauma and prior deployment

Variable	Beta	SE	t value	p value
Intercept	6.2	0.3	19.8	2.7e-77
Age	-0.005	0.01	-0.4	0.7
Baseline pNPY	0.3	0.02	16.3	7.6e-53
Combat Exposure	-0.2	0.05	-3.8	0.0001
Childhood Trauma	0.3	0.09	2.9	0.004
Prior Deployment	-0.1	0.08	-1.2	0.2

- N=1744 Marines
- Combat exposure prediction improves with inclusion of childhood trauma
- Adjusted for baseline pNPY levels, ancestry, and prior deployment status

Linear Regression

Baseline pNPY levels do not predict post-deployment PTSD

Count Model:

Variable	Beta	SE	z value	p value
Intercept	2.8	0.23	12.1	5.3e-34
pNPY	0.007	0.01	0.6	0.5
Age	-0.006	0.007	-0.9	0.4
BMI	0.01	0.008	1.5	0.1

Zero-Inflated Model:

Variable	Beta	SE	z value	p value
Intercept	-1.1	0.7	-1.5	0.1
pNPY	-0.01	0.03	-0.4	0.7
Age	-0.005	0.02	-0.3	0.8
BMI	-0.01	0.02	-0.5	0.6

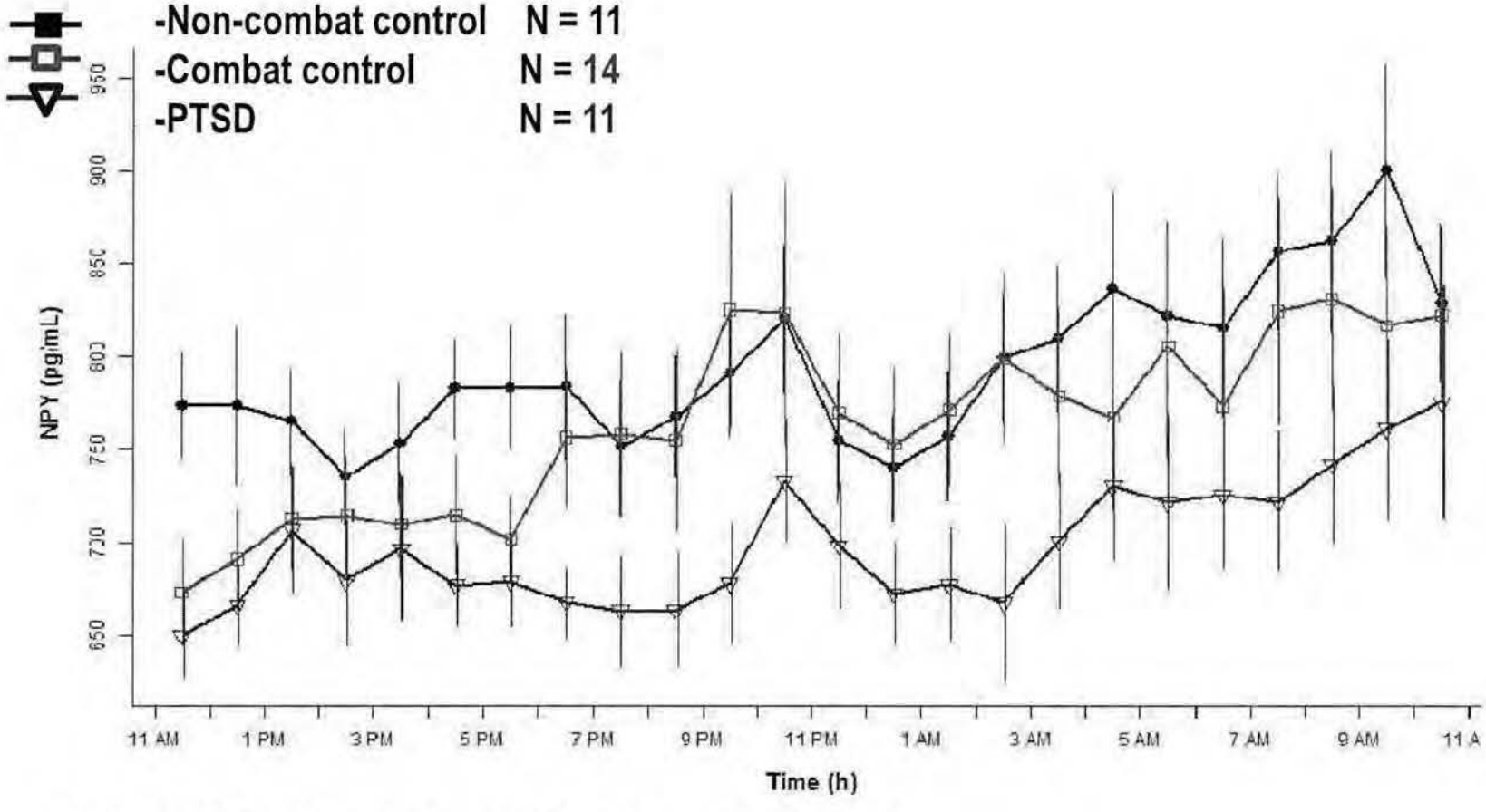
- N=1813 Marines
- CAPS Summary Score
- Adjusted for ancestry

ZINB Model

Cerebrospinal Fluid NPY Concentration, Anxiety, Temperament, and PTSD Status

- Preclinical studies
  - Lower NPY1R and NPY5R, but not NPY or NPYR2 associated with anxious temperament in primates (Rosebloom et al., 2014)
  - Intranasal NPY reverses anxiety-like behavior in rodent PTSD model (Serova et al., 2013) & Early intervention with intranasal NPY prevents prolonged-stress triggered impairments (Laukova et al., 2014)
  - Adaptive responses in CSF NPY expression following stress have been observed (McGuire et al., 2011; Makino et al., 2000 & Thorsell et al., 1998)
- Humans: Lower CSF NPY concentrations in PTSD compared to non-combat (Sah et al., 2011) and combat controls (Sah et al., 2014)

Low CSF NPY levels in PTSD



Using linear mixed-effect models: **Differences in NPY-CSF concentrations were statistically significant** [*p*-value for existence of group effect 0.0234; the difference in levels between healthy civilian volunteers and PTSD subjects was statistically significant ($p = 0.012$) but deployed healthy subjects were not statistically significantly different from either of the other two groups

Summary

- Longitudinal data in Marines confirms outcomes from cross sectional studies; results show that plasma NPY concentrations are determined by:
  - Genotype
  - Childhood Trauma
  - Combat trauma exposure
  - Pre-deployment plasma NPY does not predict PTSD outcome

- 24-h serial CSF NPY analysis replicates prior single time point studies showing lower CSF NPY concentrations in PTSD compared to combat controls and civilians

Unanswered questions/future directions

- Is low brain (amygdalar?) NPY a pre-trauma risk factor for PTSD development, or is trauma related adaptive changes in NPY a primary cause?
- Can brain NPY production be inferred from genotype determined NPY levels periphery?
- Are molecular mechanisms of stress-related adaptive change in the periphery (as has recently been described in adrenal NPY expression (Wang & Whim 2014) the same those in the CNS?

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Marine Resiliency Study

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- Caroline Nievergelt, Ph.D., Co-I, UCSD
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- Nilima Biswas Ph.D. UCSD
- Kuixing Zhang Ph.D., UCSD

Serial CSF Study

- Richard Hauger M.D., Co-I UCSD/CESAMH
- Tobias Moeller-Bertram M.D., Co-I, UCSD/CESAMH
- Piyush Patel, M.D., Co-I, VA/UCSD
- CSF Study Data Collection Staff

Serial CSF Statistics/NPY Assay

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- Paul Clopton Ph.D. VASDHS

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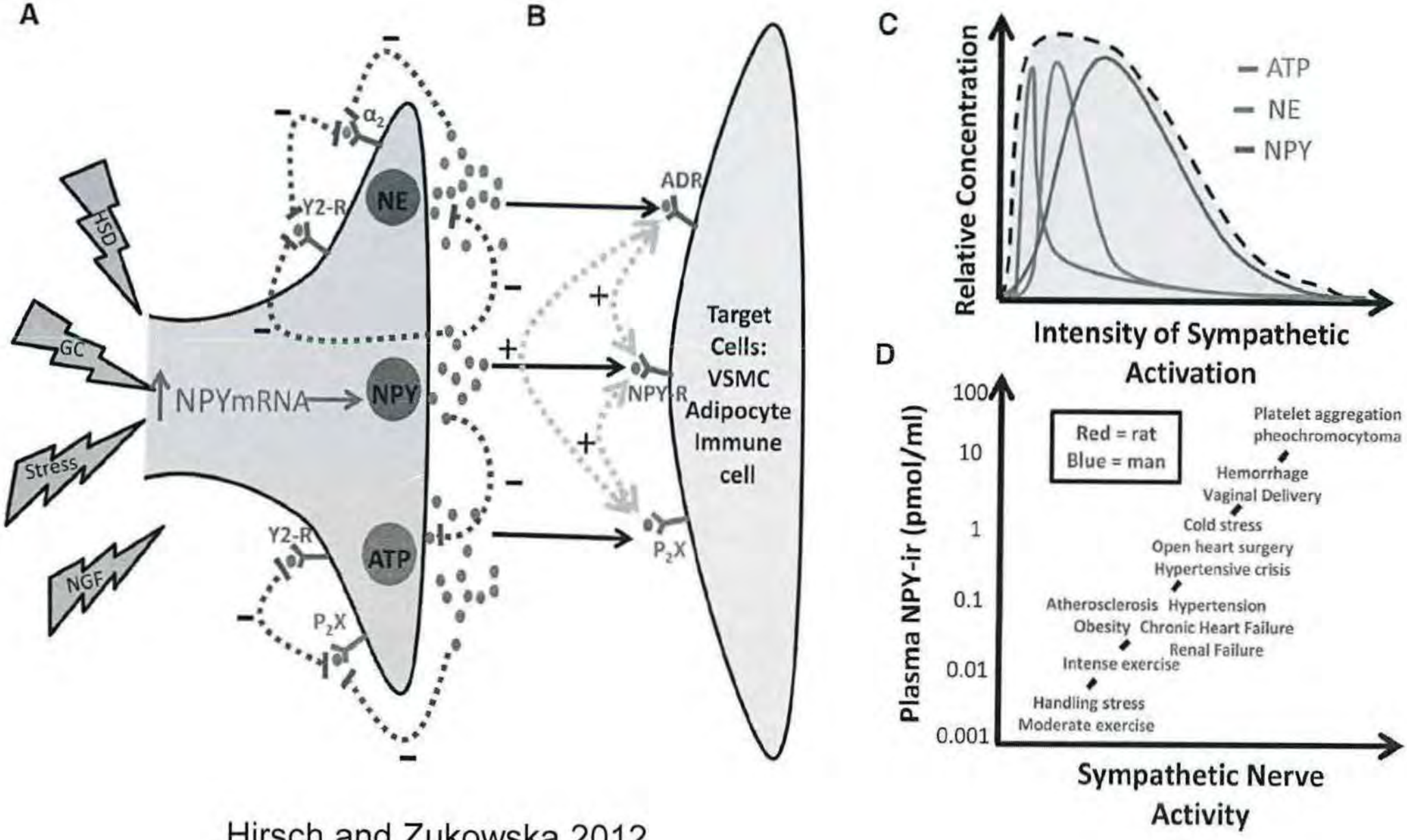
- George Chrousos M.D., AUMS, Consultant



Hirsch and Zukowska 2012

Developing MEG and DTI Markers for PTSD

Mingxiong Huang, Ph.D.
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VA San Diego Healthcare System, San Diego, CA

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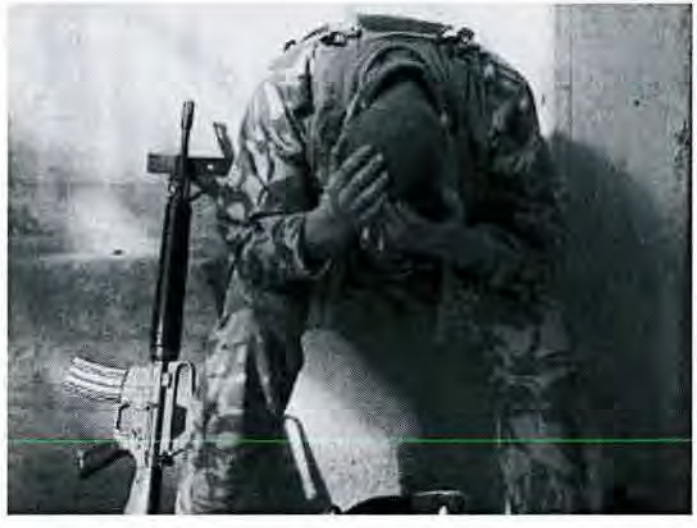
Post-Traumatic Stress Disorder: PTSD

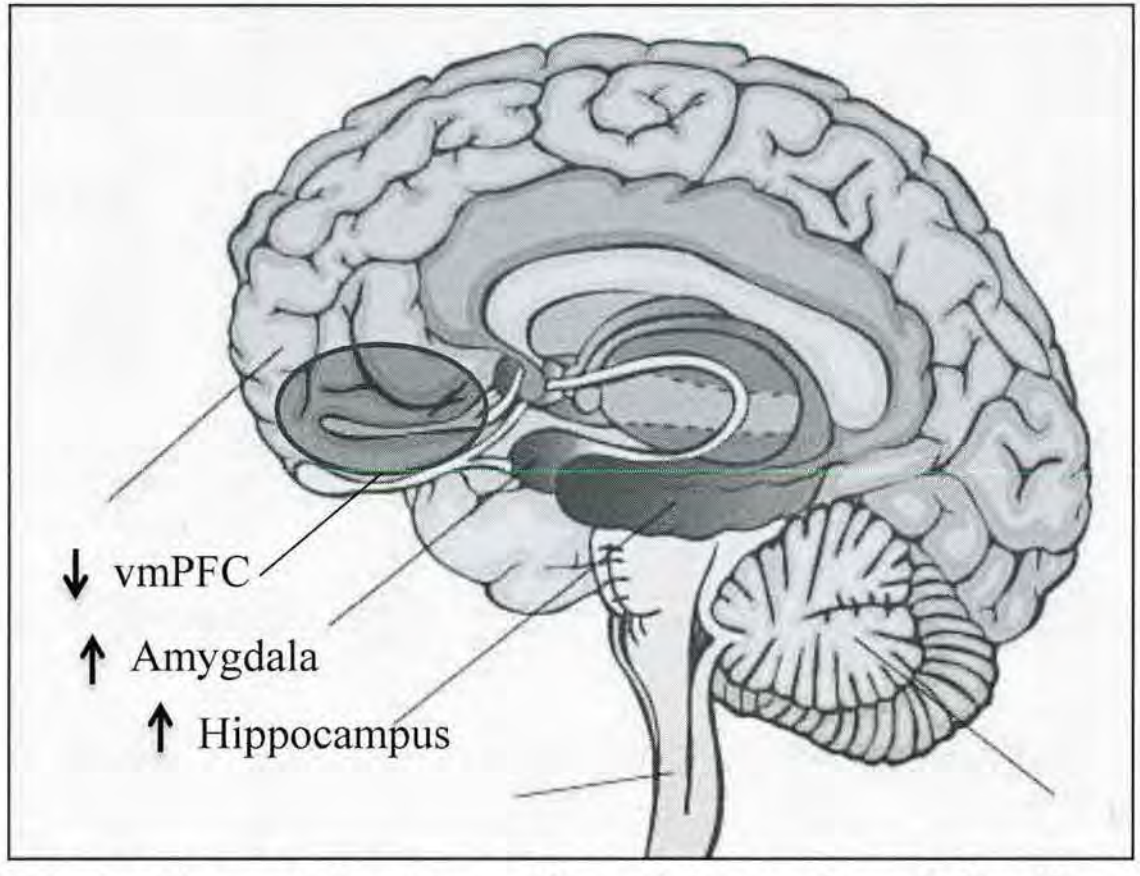
OEF/OIF/OND Military Service Members and Veterans in the U.S.



Devastating Earthquake and Tsunami, Japan, 2011

Tiananmen Square Massacre, Beijing, China, 1989

The neurocircuitry of PTSD



- PET and fMRI studies using emotional (e.g., fearful) stimuli and resting-state design showed (e.g., Shin LM and Liberzon I. Neuropsychopharmacology 35: 169-191, 2010):
- Hyper-responsive Amygdala
- Hyper-responsive Hippocampus
- Hypo-responsive ventromedial prefrontal cortex (vmPFC)

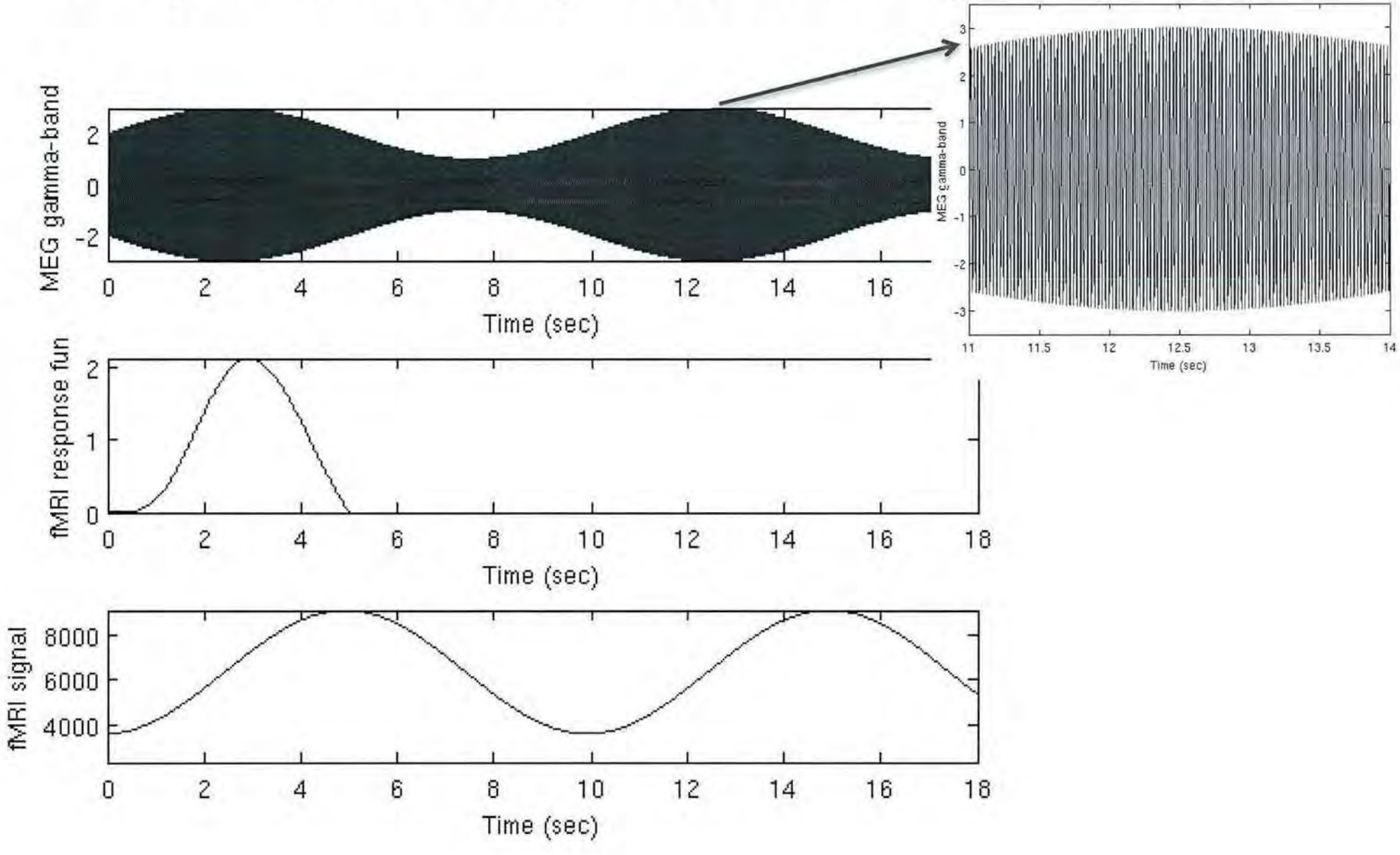
Limitations of PET and fMRI Studies for PTSD

- Low temporal resolutions (mins to secs)
- Indirect measures of neuronal activity

Questions to be Addressed by Current MEG Study

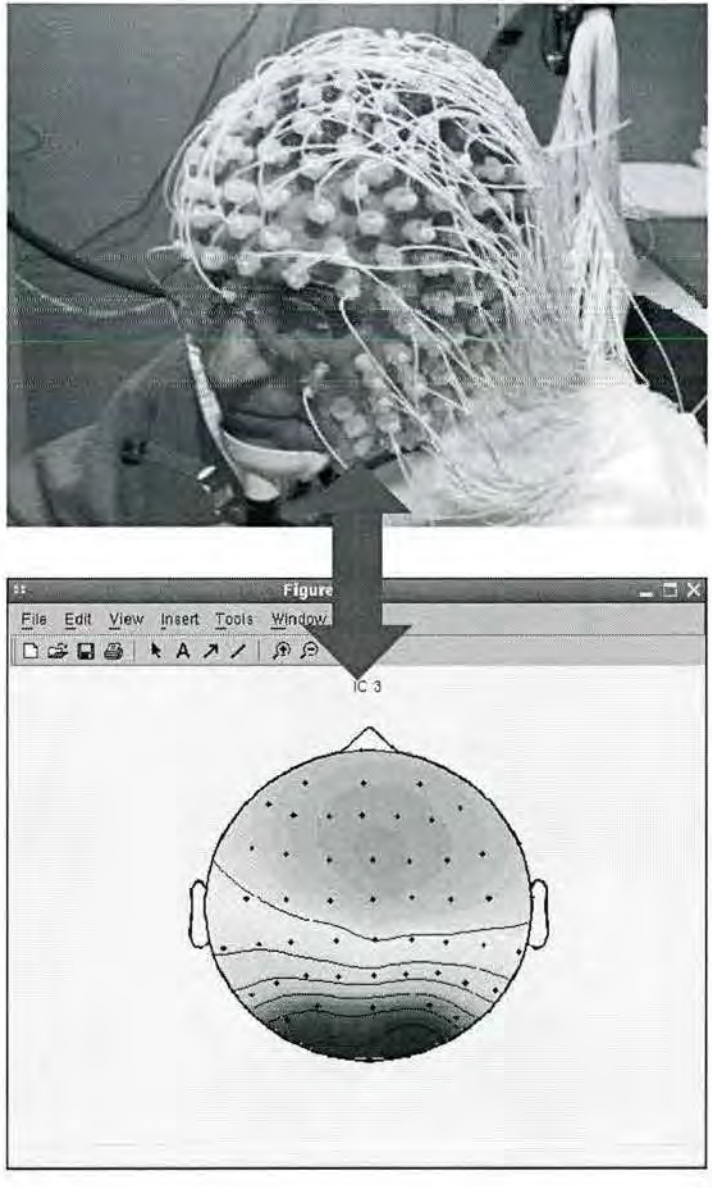
- Is MEG source imaging able to directly detect abnormal electromagnetic signals in PTSD neurocircuitry?
- If yes, at what frequency bands?
- How similar are the MEG source imaging findings to the PET/fMRI findings in PTSD?

Why MEG Society should not Ignore fMRI?



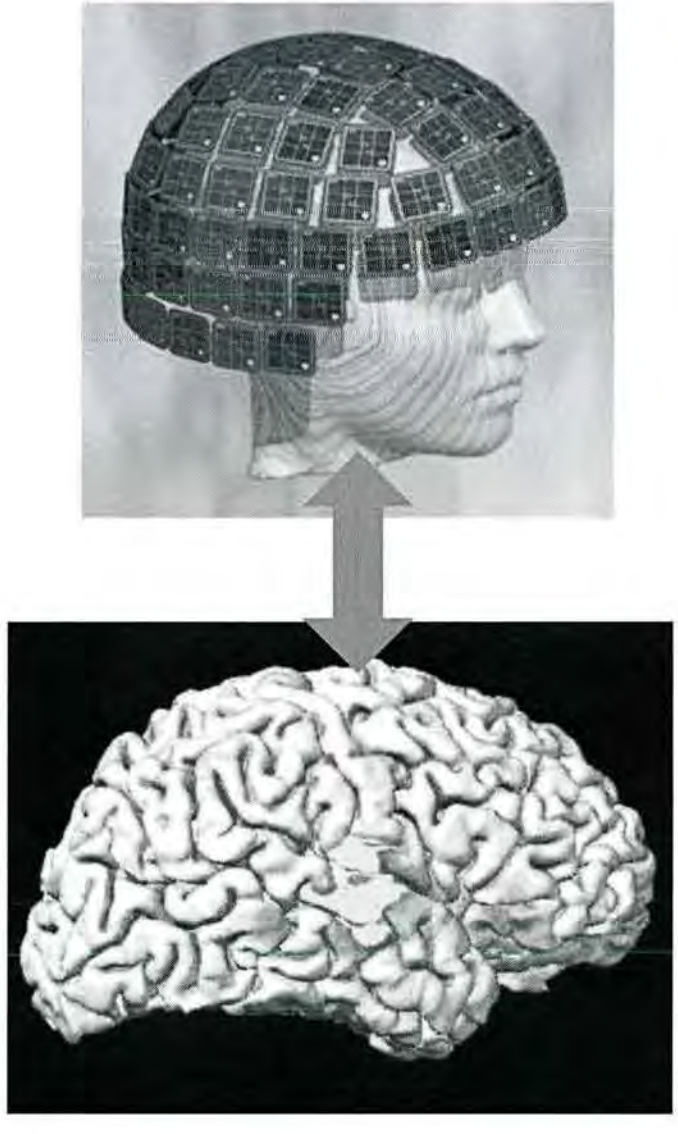
EEG vs. MEG Technology, both with millisecond temporal resolution

EEG



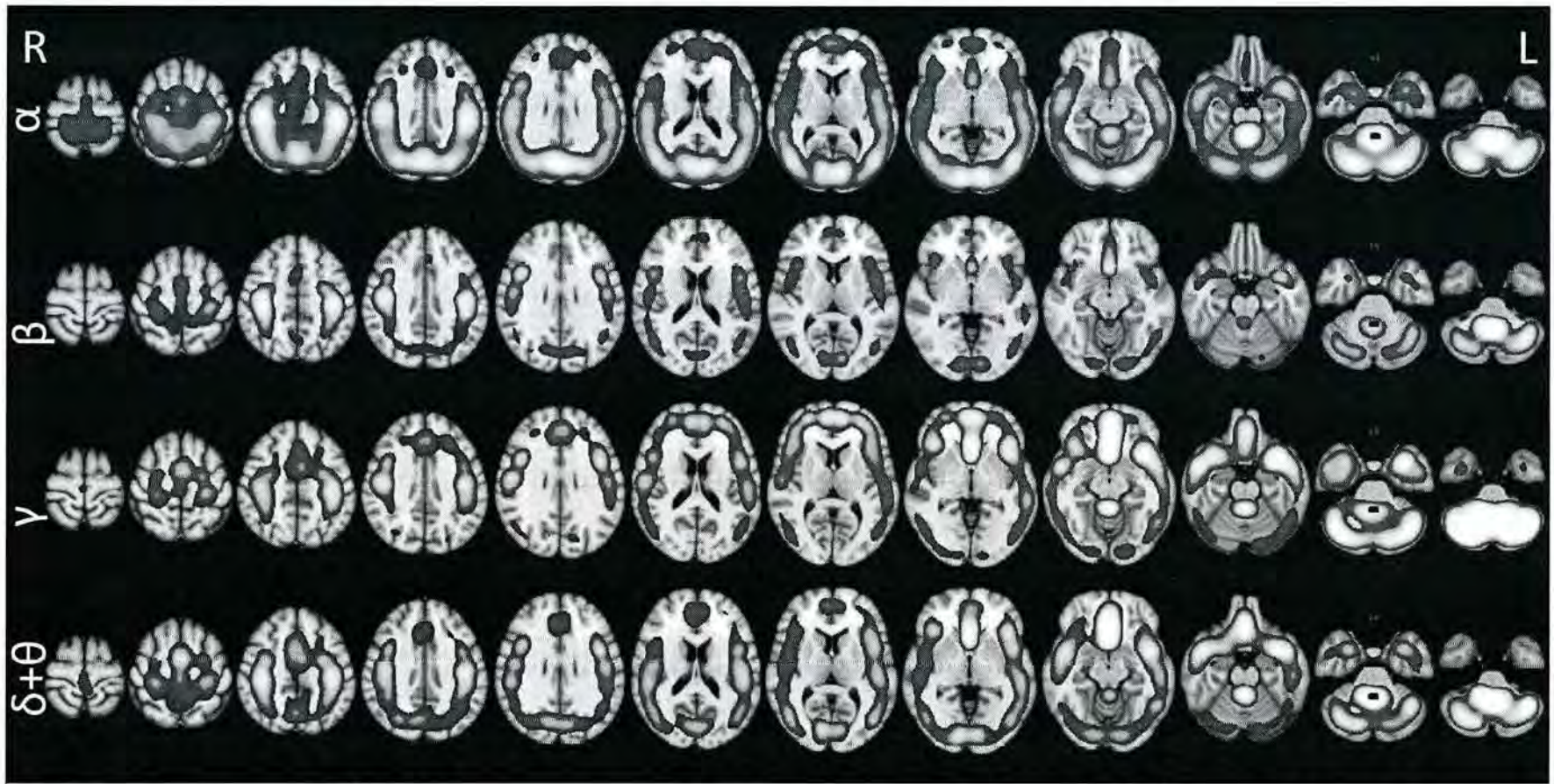
MEG

MEG SQUID Sensor Array



MEG Source Magnitude Images of Brain Rhythms for Different Frequency Bands (α: 8-12 Hz, β: 15-30 Hz, γ: 30-80 Hz, δ+θ: 1-7 Hz)

Huang et al., NeuroImage, 84: 585-604, 2014 (Fast-VESTAL)



Whole brain rs-MEG source-amplitude images averaged from 41 healthy subjects in MNI-152 atlas coordinates from **Fast-VESTAL** in alpha (1st row), beta (2nd row), gamma (3rd row), and low-frequency (delta plus theta, 4th row) bands.

MEG protocol for imaging the neurocircuitry of PTSD

- Resting-state MEG recording with eyes-closed
- 25 active-duty and Veterans diagnosed with PTSD (CAPS total: 41-81)
- 30 Healthy Controls
- MEG source imaging for different frequency bands
- Hypothesis 1: hyper-activity in amygdala and hippocampus
- Hypothesis 2: hypo-activity in ventromedial prefrontal cortex (vmPFC)

Huang et al., Voxel-wise resting-state MEG source magnitude imaging study reveals neurocircuitry abnormality in active-duty service members and veterans with PTSD. NeuroImage: Clinical (in press) 2014

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MEG data processing

- MaxFilter and ICA for removing noise and artifacts
- Data were divided into 2.5 sec epochs
- Apply DC correction, band-pass filter for alpha (8-12 Hz), beta (15-30 Hz), gamma (30-80 Hz), high-gamma (80-150 Hz), and low-frequency (1-7 Hz) bands.
- Calculate sensor waveform covariance matrix
- Run Fast-VESTAL to obtain voxel-wise source images
- Run spatial smoothing, then registered to MNI space.
- Perform logarithm transformation
- Voxel-wise comparison between PTSD and healthy controls
- Statistical analysis and correct for family-wise error

Huang et al., NeuroImage: Clinical (in press) 2014

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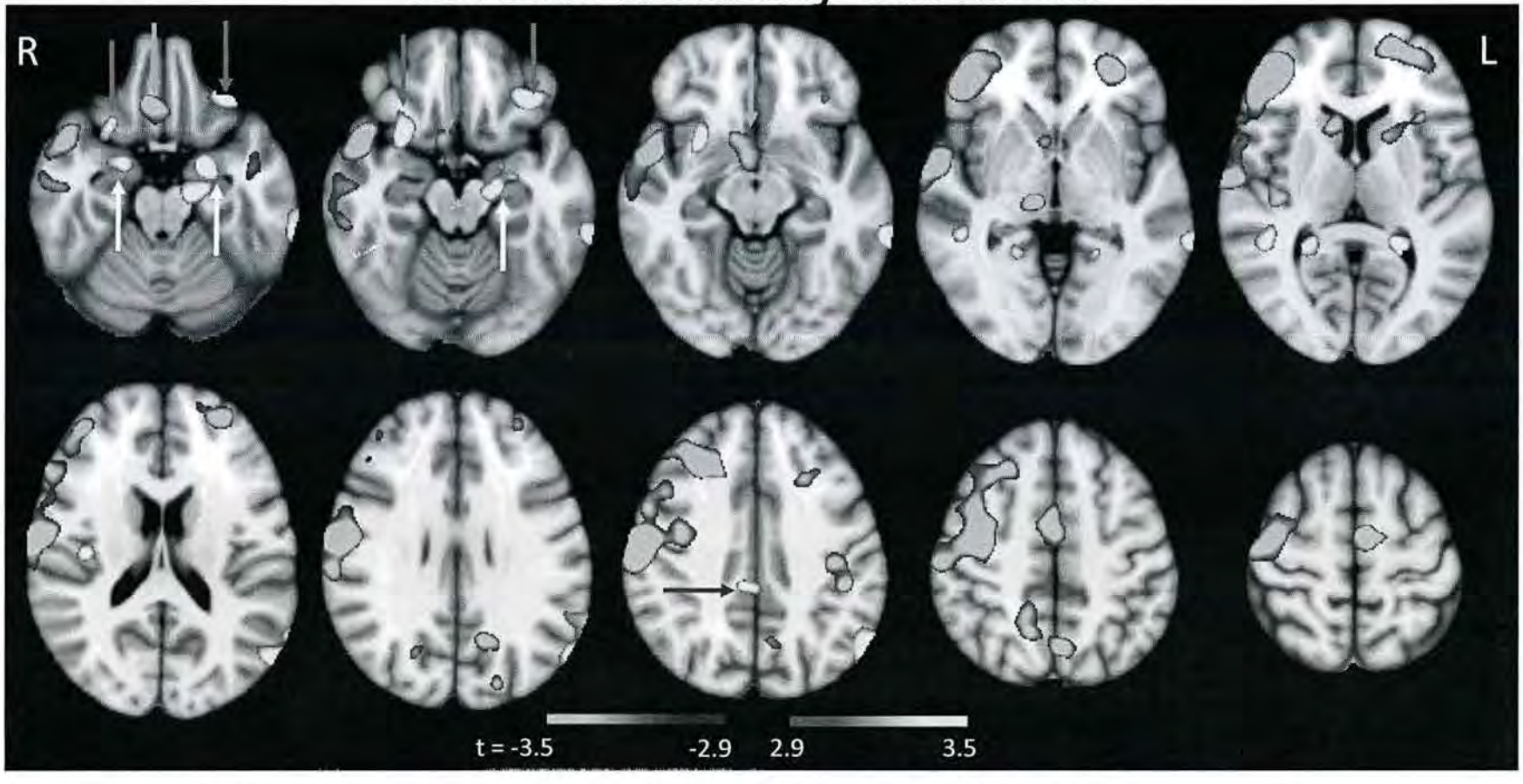
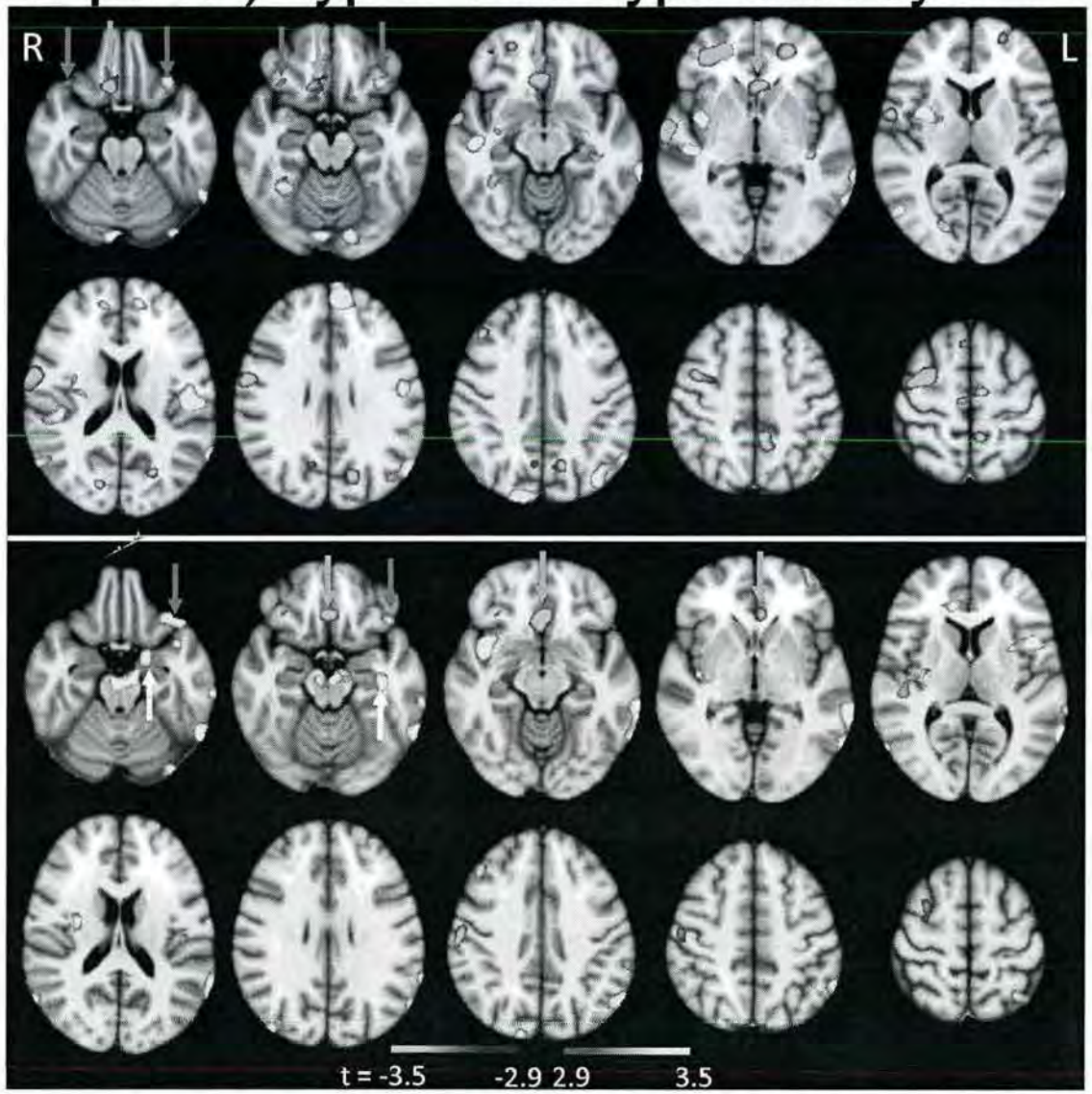
MEG Beta-band hyper- and hypo-activity in PTSD versus healthy controls



•Hyper-activity: L+R Amygdala (white arrows), L hippocampus, L+R posterolateral OFC (magenta arrows), R insular cortex, PCC (brown arrow), etc.
•Hypo-activity: vmPFC (green arrows), L+R dlPFC, precuneus cortex, L+R frontal poles, L temporal poles, etc.

Huang et al., NeuroImage: Clinical (in press) 2014

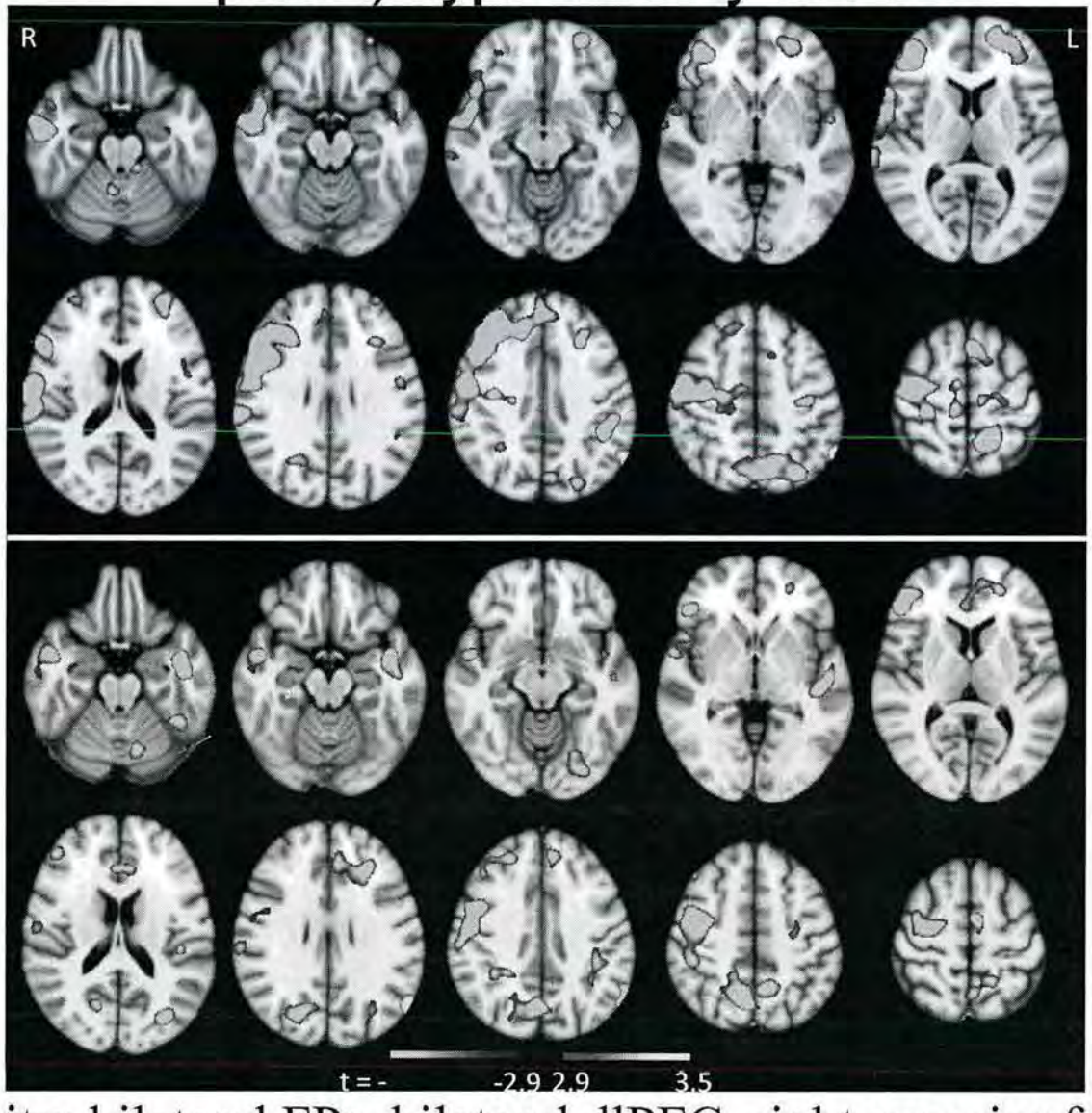
MEG gamma-band (upper panel) and high gamma band (lower panel) hyper- and hypo-activity in PTSD



•Hyper-activity: L+R Amygdala (white arrows), L hippocampus, L+R posterolateral OFC (magenta arrows), L+R insular cortex, dmPFC, etc.

•Hypo-activity: vmPFC (green arrows), L dlPFC, precuneus cortex, etc.

MEG alpha-band (upper panel) and low-freq band (lower panel) hypo-activity in PTSD



Hypo-activity: bilateral FPs, bilateral dlPFC, right superior frontal gyrus, bilateral anterior temporal lobes, bilateral precuneus cortices, and bilateral sensorimotor cortices.

PTSD Symptoms (CAPS) Correlating with MEG Source Magnitude

- Positively correlated with MEG left amygdala (beta band, $r = +0.51$, $p < .05$).
- Positively correlated with left posterolateral OFC (beta band, $r = +0.55$, $p < .05$)
- Negatively correlated with vmPFC (beta band, $r = -0.58$, $p < .01$; gamma band, $r = -0.63$, $p < .01$; and high-gamma band, $r = -0.60$, $p < .01$).
- Negatively correlated precuneus (alpha band, $r = -0.48$, $p < .05$)
- Using the MEG source magnitude from the above areas, support vector machine (SVM) correctly classified PTSD patients with 93% accuracy, and healthy controls with 95% accuracy.

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Resting-state fMRI findings in PTSD

Yan et al., Neuroscience Letters. 547: 1-5, 2013

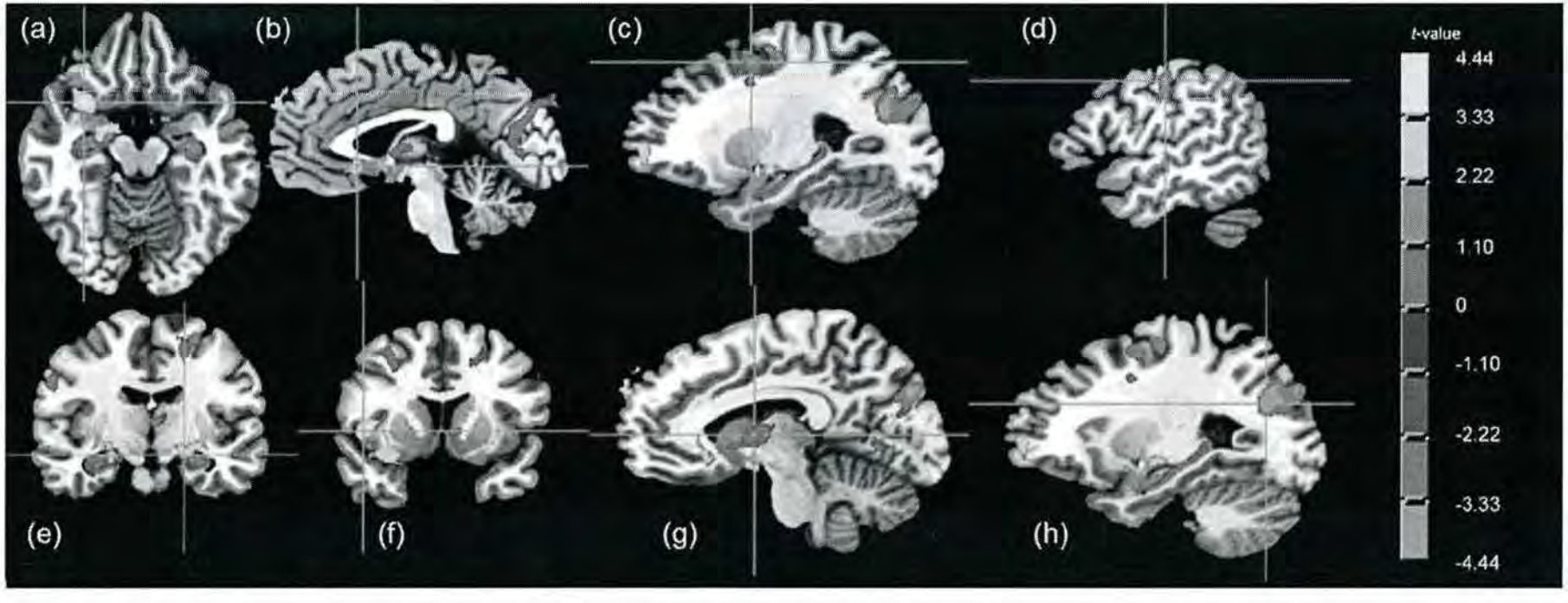


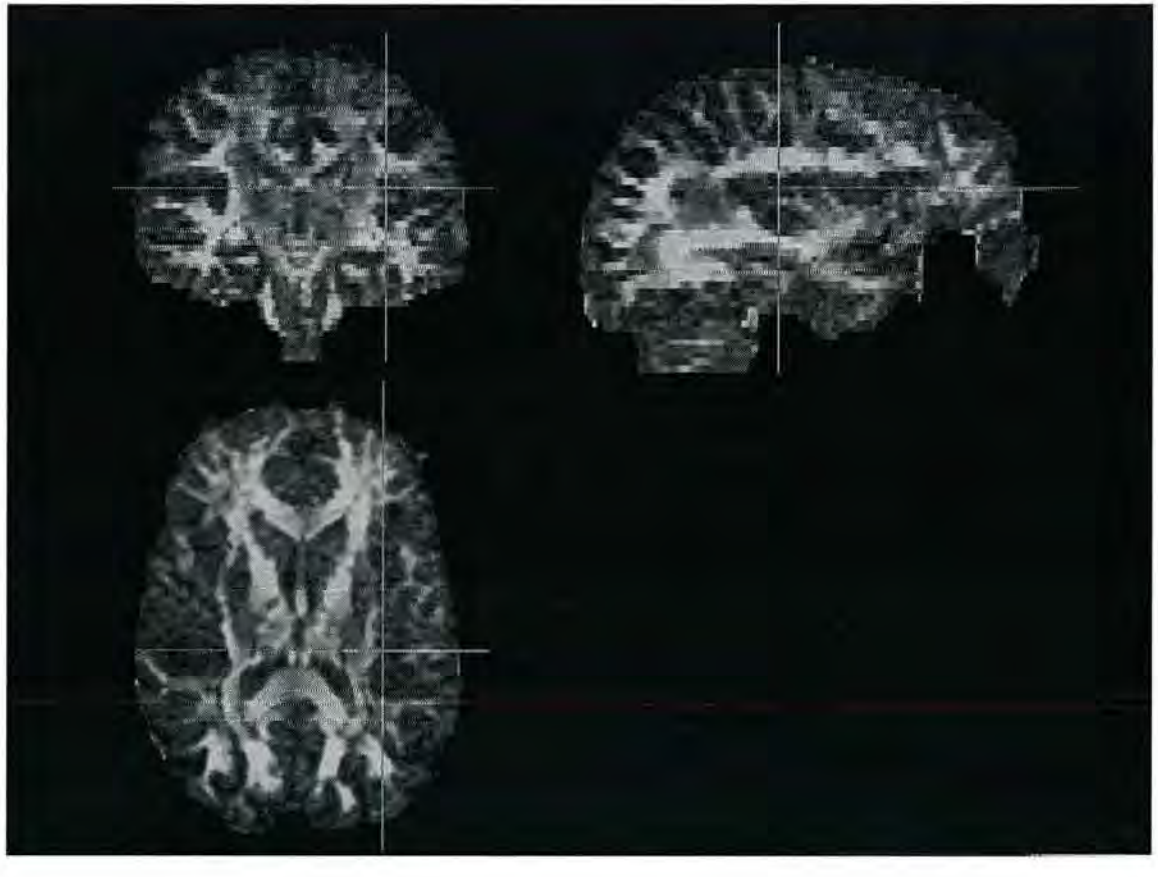
Fig. 1. Brain regions showing significant group differences between PTSD and controls in terms of magnitudes of spontaneous activity. The crosshairs are focused at the following brain regions: (a) orbital frontal gyrus, (b) anterior cingulate cortex, (c) superior frontal gyrus, (d) dorsal lateral prefrontal cortex, (e) amygdala, (f) insula, (g) thalamus and (h) precuneus. Warm colors (red and yellow) represent increased spontaneous activity in the PTSD group compared to the control group, whereas cold color (blue) represents decreased spontaneous activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Diffusion Tensor Imaging (DTI)

- DTI is an MR imaging technique based on the Brownian motion of water through tissues
- It measures how easy that water molecules move along the direction of white matter fibers versus the directions perpendicular to the fibers.
- TBI causes tissue shearing in the white matter fibers that leads to reduction of DTI signal.

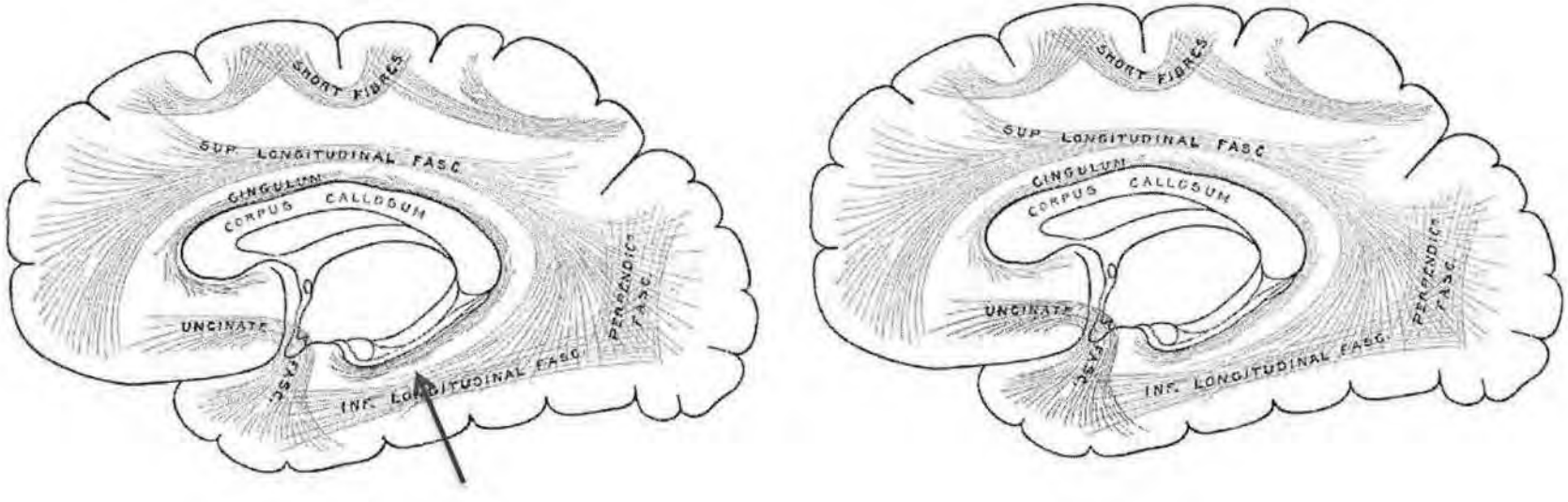
$$D = \begin{pmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{pmatrix}$$

$$FA = \frac{\sqrt{3\left[(\lambda_1 - \bar{\lambda})^2 + (\lambda_2 - \bar{\lambda})^2 + (\lambda_3 - \bar{\lambda})^2\right]}}{\sqrt{2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}}$$



Preliminary abnormal DTI findings in PTSD

- Reduced FA in bilateral temporal-portions of the posterior cingulum, consistent with findings in Fani et al., 2012
- Reduced FA in bilateral Uncinate Fasciculus



Fani et al., Neuropsychopharmacology (2012) 37: 2740–2746

Summary

- In MEG beta and gamma bands, PTSD showed hyperactivity in amygdala, hippocampus
- PTSD showed hypoactivity in vmPFC, dlPFC, precuneus, frontal poles, anterior temporal lobes
- New finding: hyperactivity from posterolateral OFC
- MEG abnormal activity correlated with PTSD symptom scores.
- MEG findings are similar to fMRI findings, but MEG offers markedly more information in terms of new abnormal areas, frequency-bands, etc.

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MRS-II MEG Study

- Funding: Marine Resilience Study-II (MRSII PI: Baker, Exploratory MEG Project PI: Huang; Co-I: Victoria Risbrough; MRS-II; Navy BUMED contract #N62645-11-C-4037), Merit Review Grant from the US Department of Veterans Affairs (PI: Huang, NURC-022-10F).
- Investigator Collaboration: VA San Diego Healthcare System, and University of California San Diego

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- Victoria Risbrough Ph.D., VA & UCSD
- Caroline Nievergelt Ph.D., VA & UCSD
- Daniel O'Connor M.D., UCSD
- Robert Naviaux M.D., UCSD
- Richard Hauger M.D., VA & UCSD
- Sharon Nichols, Ph.D. UCSD
- Robert McLay M.D. Ph.D., NMCSD
- Gerald Larson Ph.D., NHRC
- MRS Research Team

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MEG and Mild Traumatic Brain Injuries / Concussions

Presented by Mingxiong Huang, Ph.D.

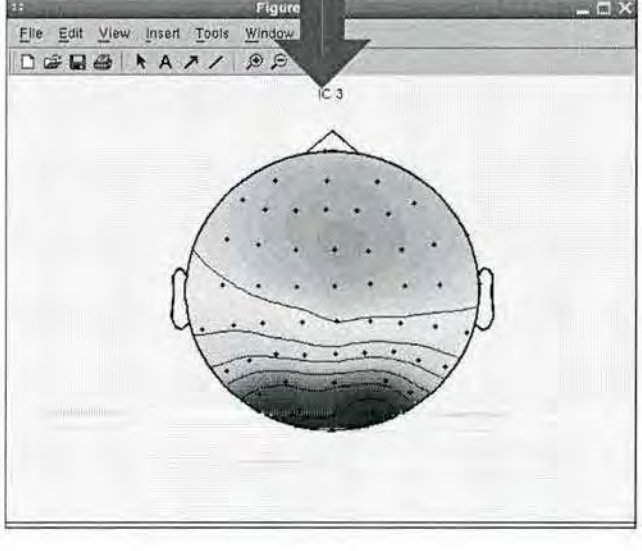
Department of Radiology, University of California, San Diego, CA

VA San Diego Healthcare System, San Diego, CA

Biomag ISACM Symposium 08/25/2014

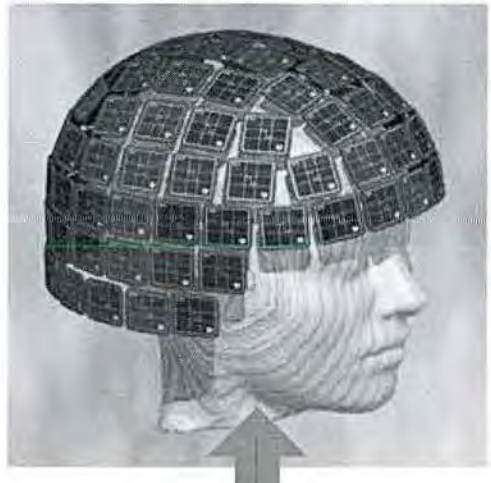
EEG vs. MEG Technology, both with millisecond temporal resolution

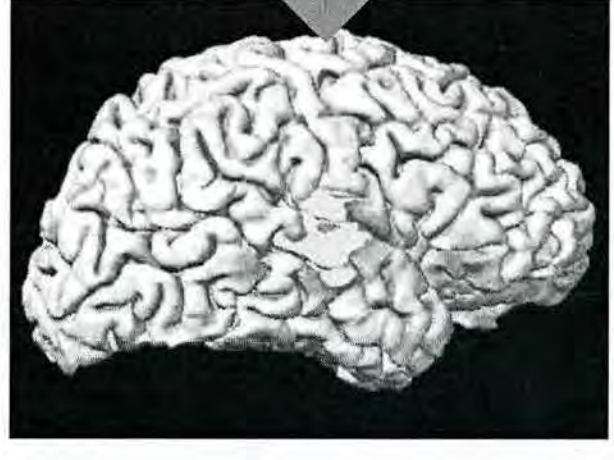
EEG



MEG

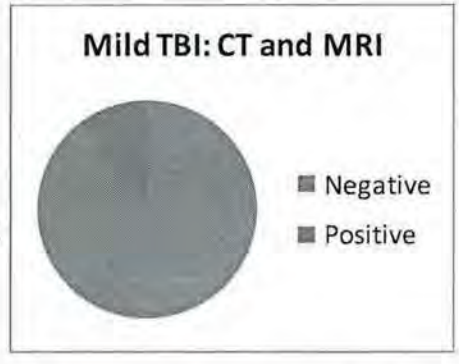
MEG SQUID Sensor Array





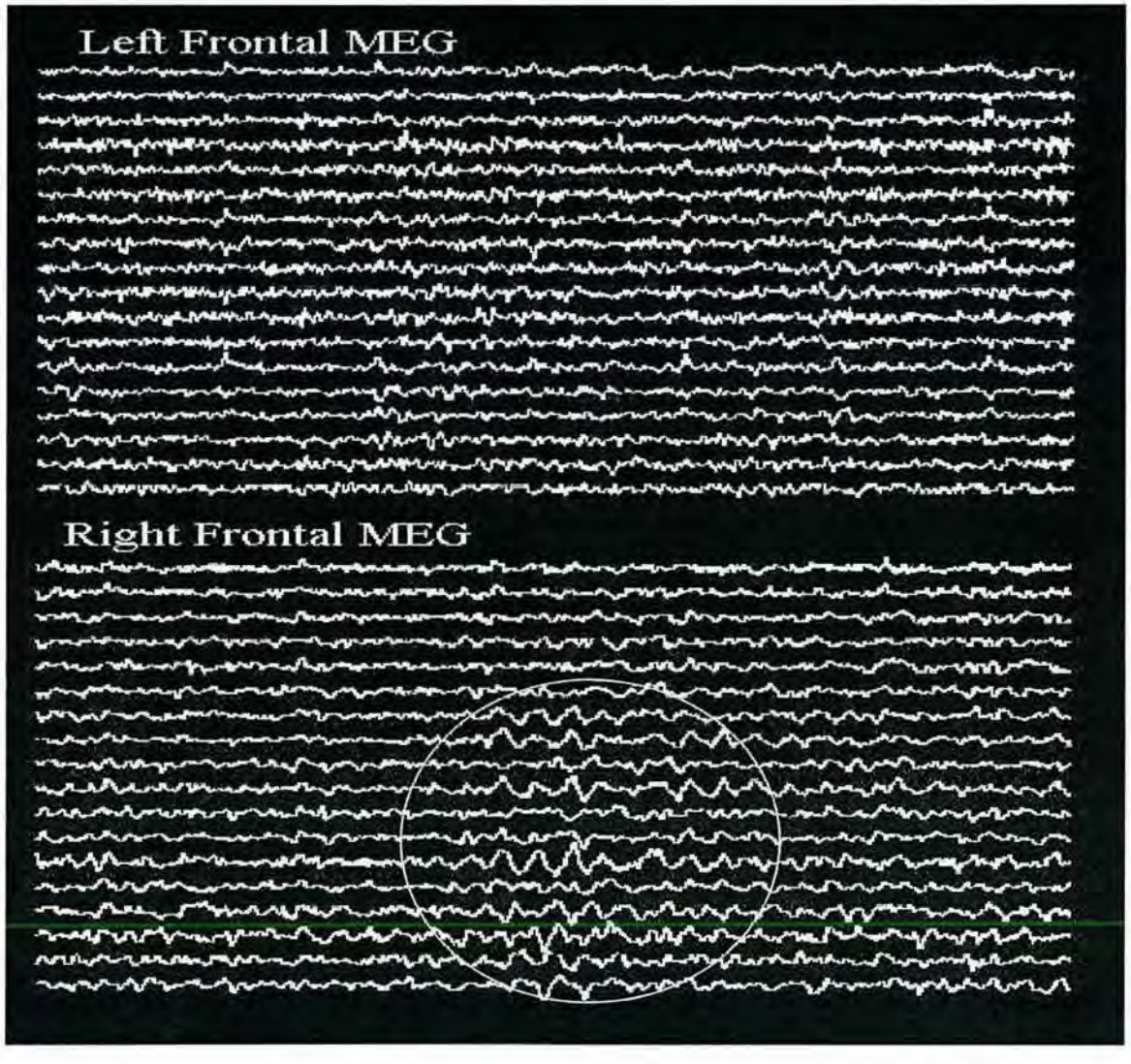
Mild TBI is often referred as *invisible* injuries: Detecting Mild TBI is Challenging using Conventional Neuroimaging Methods

- Traumatic brain injury (TBI) is a leading cause of sustained impairment in veterans, military personnel, and civilian populations.
- Mild TBI (mTBI): injuries are difficult to detect (injuries are visible on only <10% of conventional MRIs or CTs).
- Axonal injury is a leading factor in mTBI. Conventional CT and MRI are mainly sensitive to blood product, and less sensitive to axonal damage itself, hence they underestimate the presence of axonal injury, especially in mild TBI cases.
- Injured brain tissues in mTBI patients generate pathological slow-wave magnetic signal that can be measured and localized by MEG (Lewine et al., 1999, 2007).

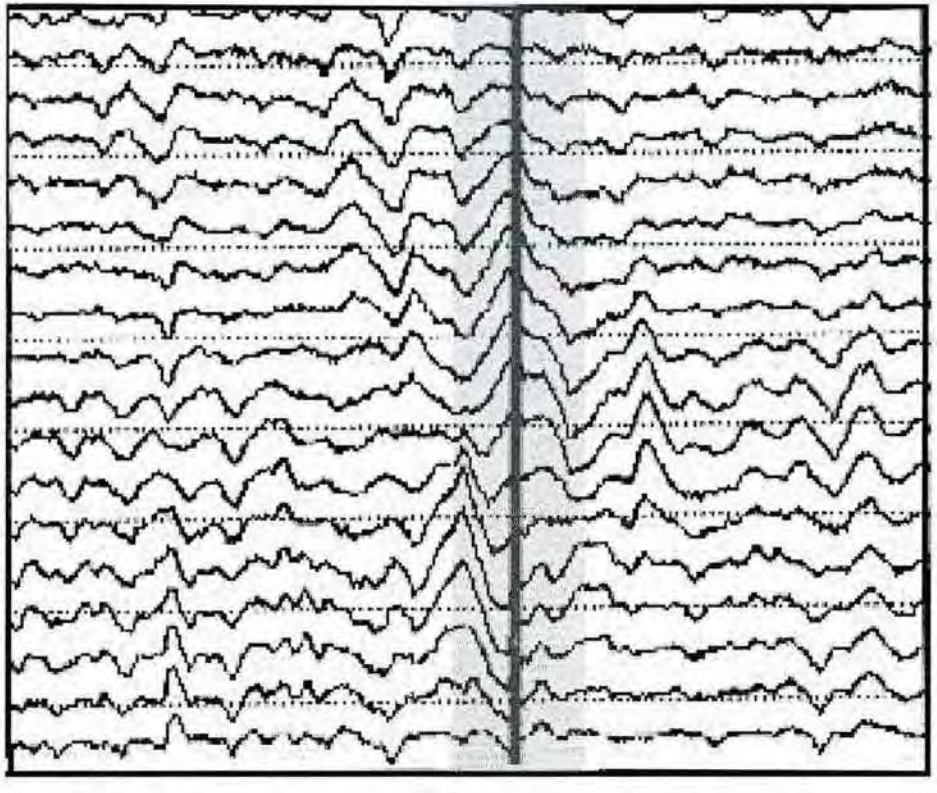


Abnormal Resting-state MEG Slow-waves in gray-matter (1-4 Hz, delta-waves) are Characteristics of Neurological Injuries in the Brain, resulting from axonal injury and/or defects in cholinergic pathways

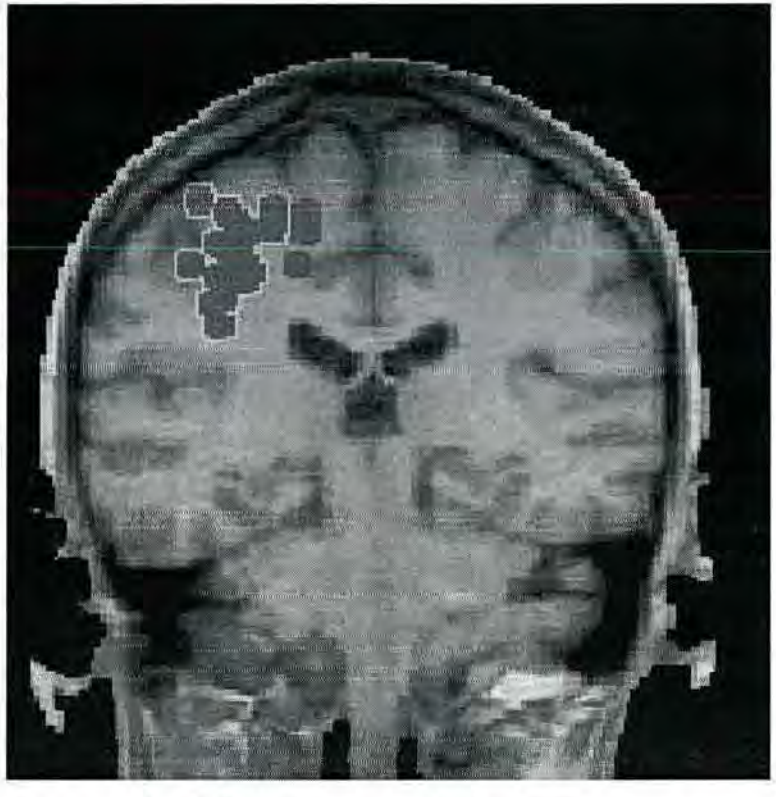
- Stroke
- Brain tumor
- Epilepsy
- Traumatic brain injury



Resting-state Slowing in mild head trauma patients with normal MRI: Dipole modeling



Dipolar Slow Wave



Mild Head Trauma

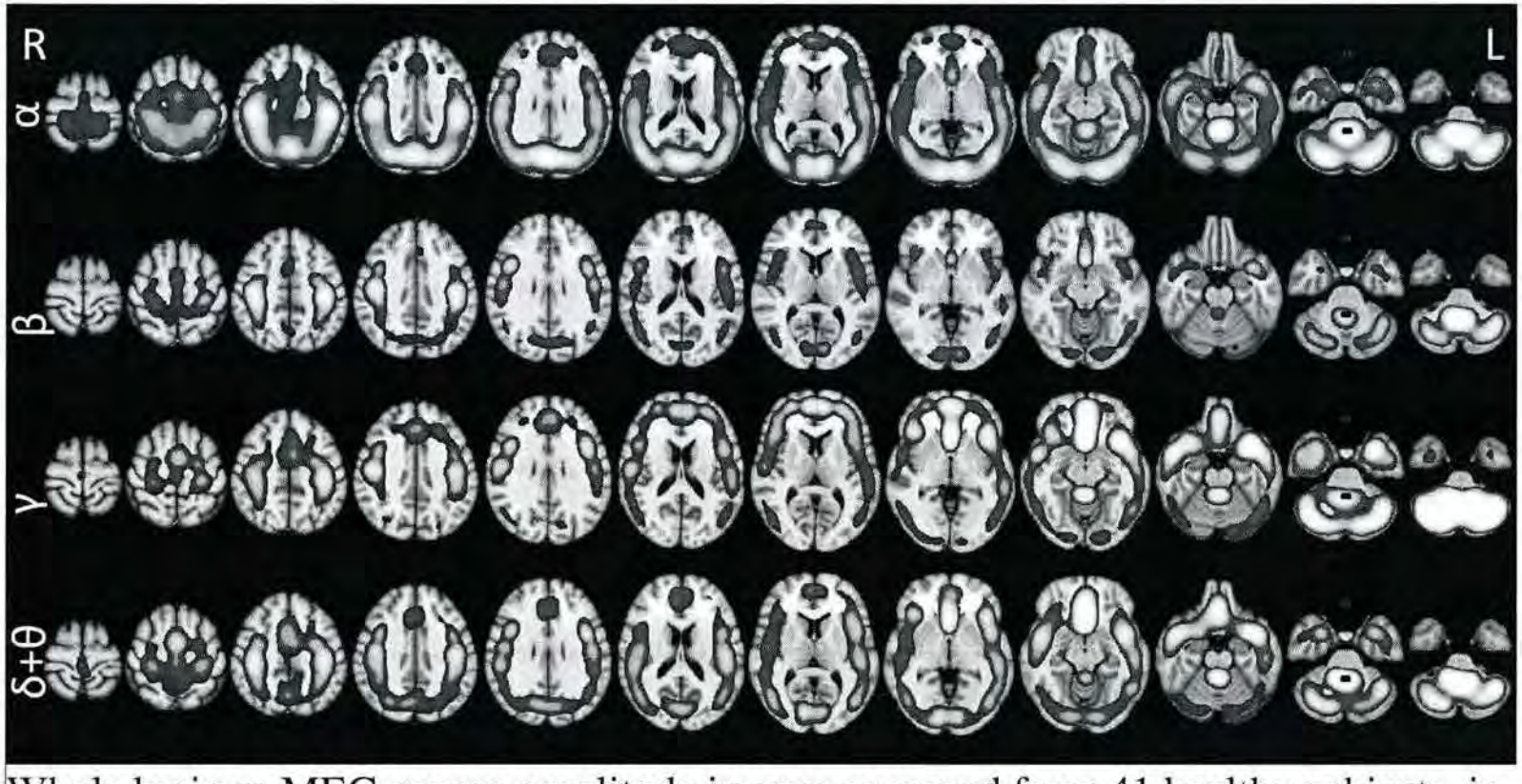
Lewine et al., 1999, AJNR Am.J.Neuroradiol. 20: 857-866

What is the neurophysiology for resting-state MEG slow-wave generation (1-4 Hz) in TBI?

- Animal studies in cats revealed the slow-waves (delta-band 1-4Hz) were due to De-afferentation in gray-matter, caused by axonal lesions in white matter tissue (Gloor et al., Neurology, 1977; Ball et al., Electroencephalogr. Clin. Neurophysiol., 1977).
- In animals, slow-waves and de-afferentation can also be generated by applying atropine that blocks or limits **cholinergic transmissions** (Schaul et al., Brain Res. 143: 475-486, 1978).

MEG Source Magnitude Images of Brain Rhythms for Different Frequency Bands (α: 8-12 Hz, β: 15-30 Hz, γ: 30-80 Hz, δ+θ: 1-7 Hz)

Huang et al., NeuroImage, 84: 585-604, 2014 (Fast-VESTAL)



Whole brain rs-MEG source-amplitude images averaged from 41 healthy subjects in MNI-152 atlas coordinates from **Fast-VESTAL** in alpha (1st row), beta (2nd row), gamma (3rd row), and low-frequency (delta plus theta, 4th row) bands.

Examining the positive detection rate of Mild TBI using automated resting-state MEG source imaging

- Resting-state MEG data (spontaneous recording with eyes-closed) were collected using the Elekta-Neuromag VectorView whole-head MEG system
- Group 1 contains 36 mild TBI patients whose injuries were caused by blast, all with persistent Post Concussion Symptoms (PCS).
- Group 2 contains the 48 mild TBI were injured with non-blast causes (i.e., motor vehicle accident, sports, and fall), all with persistent PCS.
- On average, MEG exam was done ~7 months post injury.
- Group 3 contains 79 age-matched healthy control subjects.

Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

MEG data processing

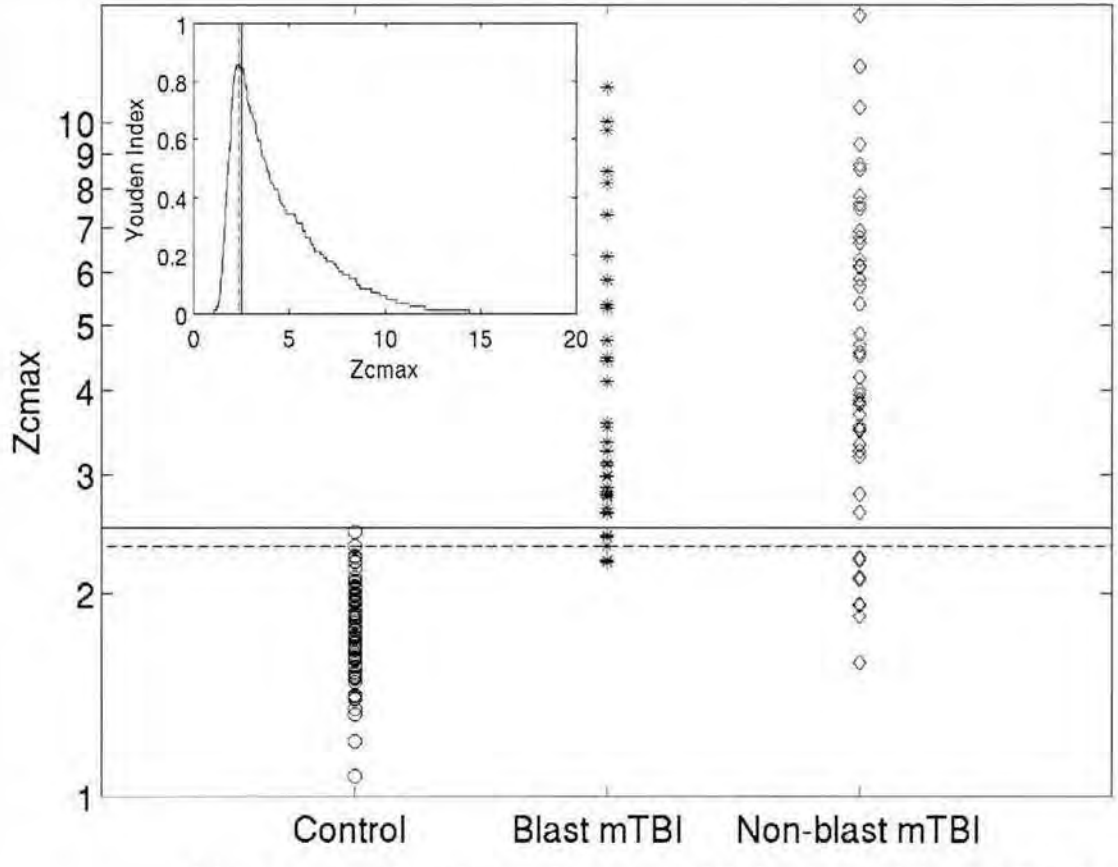
- MaxFilter and ICA for removing noise and artifacts
- Data were divided into 2.5 sec epochs
- Apply DC correction, band-pass filter 1-4 Hz
- Calculate sensor waveform covariance matrix
- Run Fast-VESTAL to obtain voxel-wise source images
- Run spatial smoothing, then registered to MNI space.
- logarithm transformation
- Compare with normative database, obtain voxel-wise Z-score maps
- Statistical analysis and correct for family-wise error

Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

MEG slow-wave positive detection rates for mTBI: voxel-wise approach

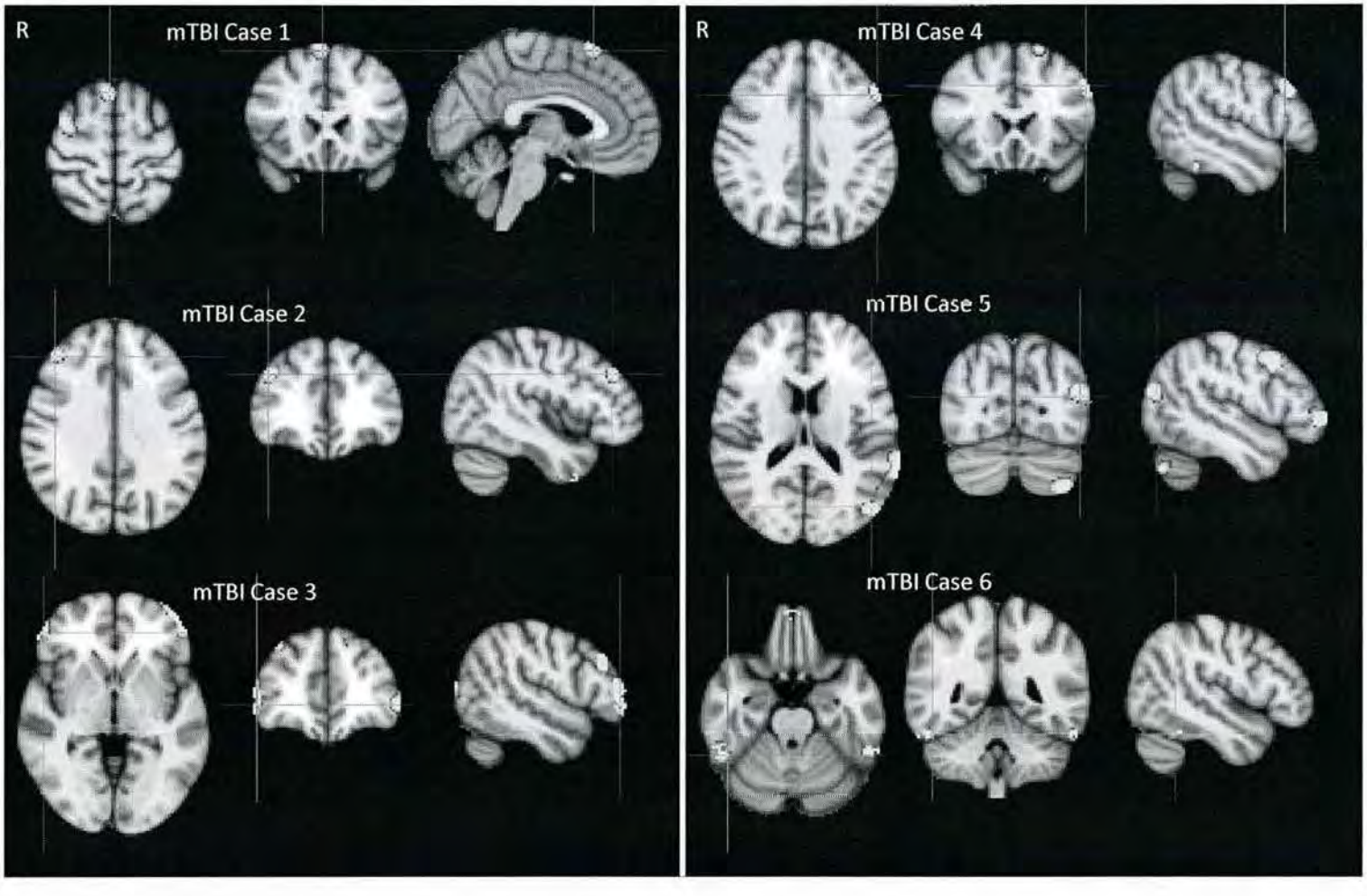
MEG positive-finding rates for different TBI groups were calculated at the threshold of 0% false-positive rate in healthy control subjects.

- In the blast mild TBI group, the MEG positive-finding rates was **86.1%**.
- In the non-blast mild TBI group, the MEG positive-finding rates was **83.3%**.
- In the combined mild TBI group (blast + non-blast), the MEG positive-finding rates was **84.5%**.



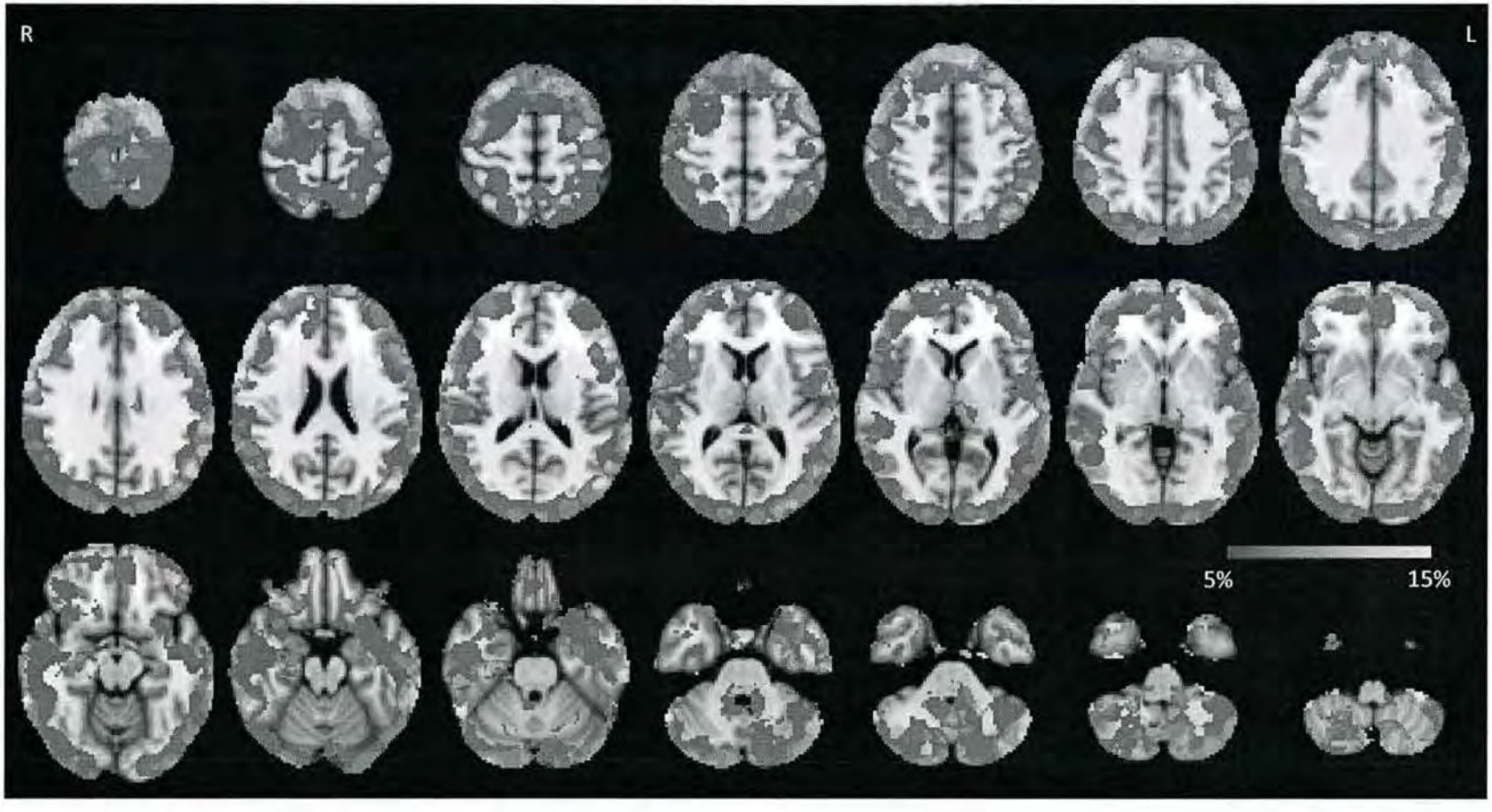
Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

Voxel-wise MEG slow-wave imaging for single mild TBI patients



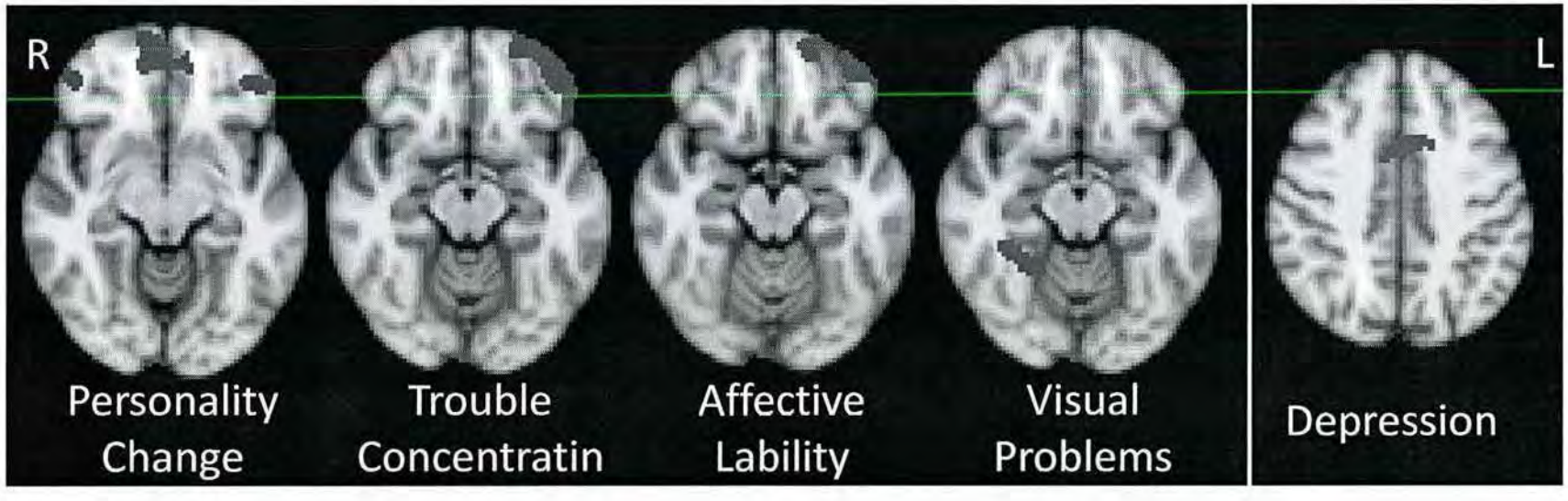
Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

Voxel-based maps showing the percent likelihood of abnormal MEG slow-wave generation



Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

MEG slow-wave source magnitude significantly correlated with PCS



Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

MEG slow-wave imaging marker for mTBI

- Positive-detection Rates: MEG slow-waves in TBI patients has ~**85**% positive-finding rate in mild TBI patients.
- The injury patterns revealed by MEG were heterogeneous, but unique to each individual mTBI patient.
- With a sufficiently large group, MEG slow-wave imaging can show brain areas that are particularly vulnerable to mTBI.
- MEG slow-wave findings correlate with post-concussive symptoms

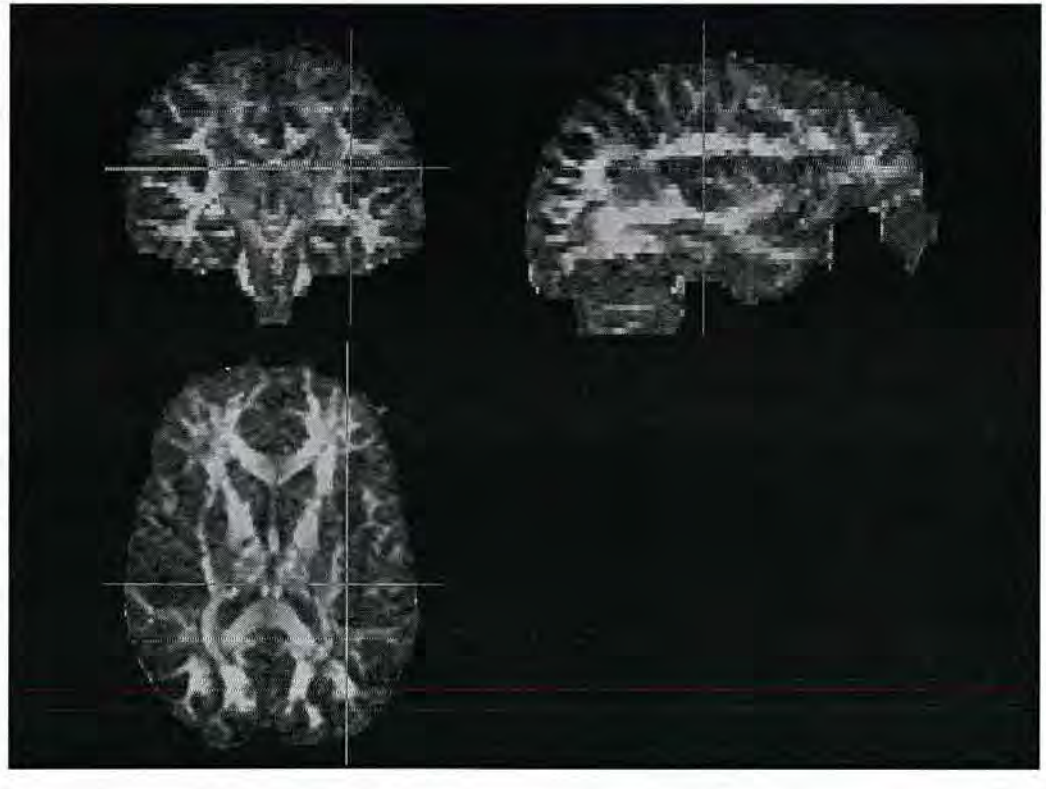
Huang et al., NeuroImage: Clinical, 2014, 5:109-119.

Diffusion Tensor Imaging (DTI)

- DTI is an MR imaging technique based on the Brownian motion of water through tissues
- It measures how easy that water molecules move along the direction of white matter fibers versus the directions perpendicular to the fibers.
- TBI causes tissue shearing in the white matter fibers that leads to reduction of DTI signal.

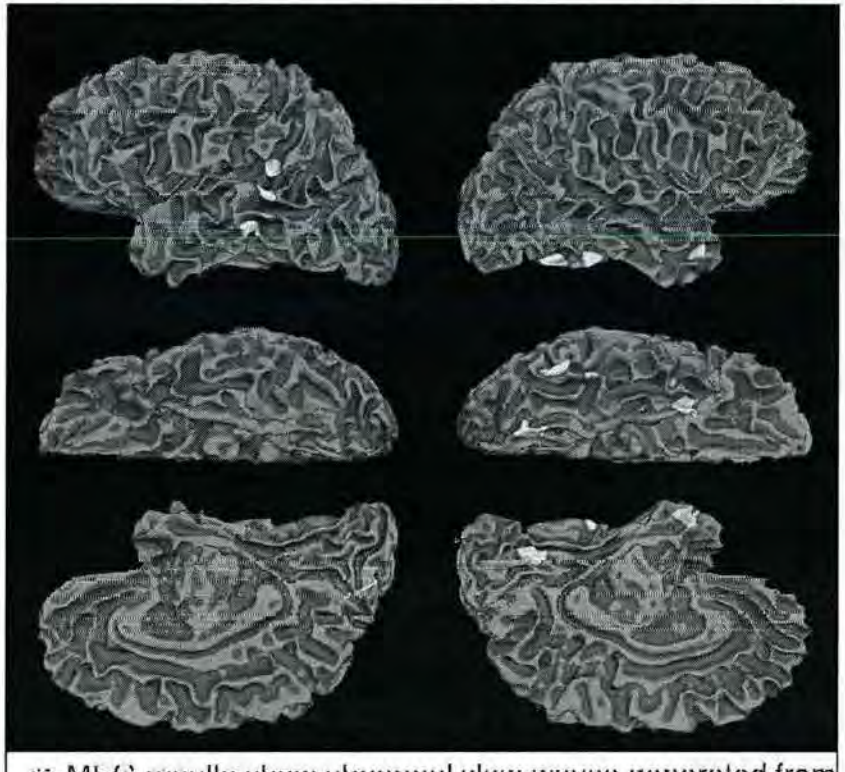
$$D = \begin{pmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{pmatrix}$$

$$FA = \frac{\sqrt{3\left[(\lambda_1 - \bar{\lambda})^2 + (\lambda_2 - \bar{\lambda})^2 + (\lambda_3 - \bar{\lambda})^2\right]}}{\sqrt{2(\lambda_1^2 + \lambda_2^2 + \lambda_3^2)}}$$

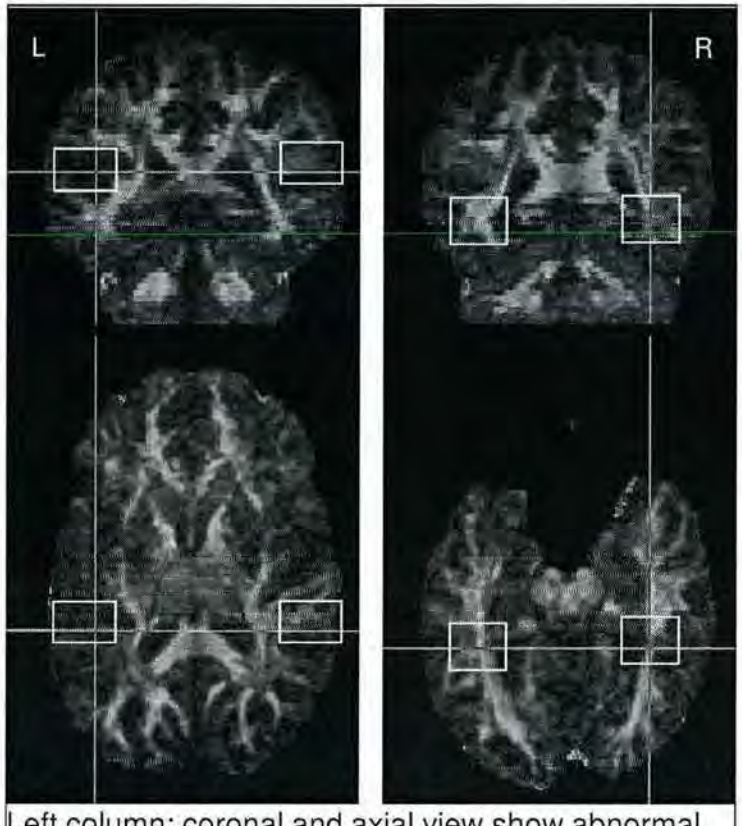


MEG-DTI Findings in Mild TBI due to Sport-related Accidents

History: 17-year old football player, 3 mTBIs. Symptoms: progressive headaches. dizziness, extreme fatigue while performing any mental task, altered sleep, memory problems, changes in speech.
Evaluation: Multiple CT & MRI scans negative.



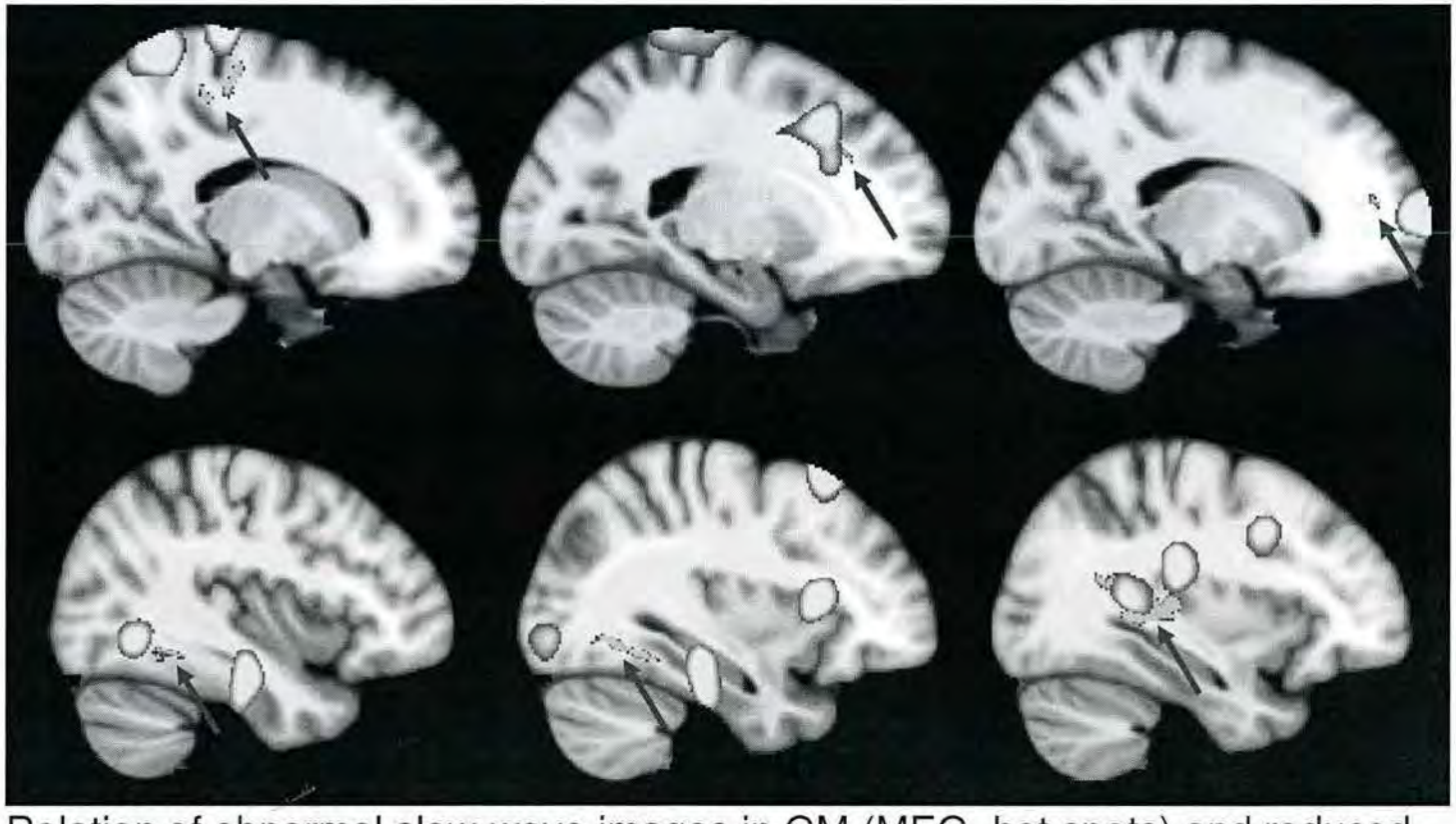
rs-MEG results show abnormal slow-waves generated from two regions in a TBI patient: 1) left column -- left lateral superior-posterior temporal region, 2) right column --- right inferior-temporal areas. Color threshold $p<0.01$.



Left column: coronal and axial view show abnormal DTI in superior-posterior temporal lobe of the left hemisphere in a TBI patient. Right column: abnormal DTI in inferior-temporal lobe as part of the inferior longitudinal fasciculus of the right hemisphere.

Huang et al., J Neurotrauma. 2009 Aug;26(8):1213-1226.

Relation of MEG slow-waves and reduced FA from DTI in mTBI patients

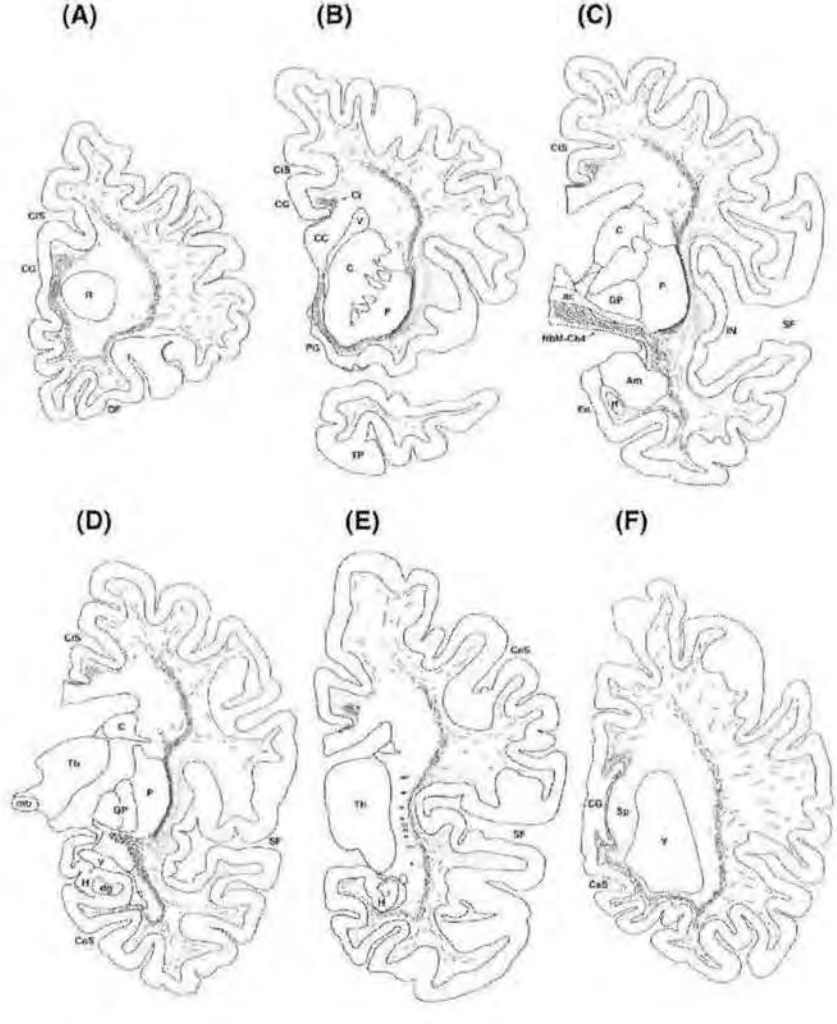


Relation of abnormal slow-wave images in GM (MEG, hot spots) and reduced FA in WM tracts (DTI, light blue clusters indicated by blue arrows) in 6 mTBI patients. All images are registered in MNI-152 atlas coordinates

Huang et al., (in preparation)

MEG Slow-wave is a sensitive measure of cholinergic blockage / limitation post mild TBI

- In animals, slow-waves and de-afferentation can also be generated by applying atropine that blocks or limits **cholinergic transmissions** (Schaul et al., Brain Res. 143: 475-486, 1978).
- Right-- Human cholinergic pathway (Selden et al., Brain 121: 2249-2257, 1998):



Summary for MEG TBI Study

- MEG slow-waves from gray-matter result from de-afferentation, due to axonal injury and/or defects in cholinergic pathways in white-matter.
- For mild TBI, automated MEG slow-wave imaging techniques (regional or voxel-based whole brain) show high positive finding rate (~85%).
- MEG slow-wave imaging findings correlate with post concussion symptom scores.
- MEG slow-wave imaging findings in gray-matter are consistent with reduced FA in white-matter tracks.

Acknowledgement

- **Support**
  - Funding: Merit Review Grant from the US Department of Veterans Affairs (PI: Huang, NURC-022-10F)., Marine Resilience Study-II (PI: Baker, Exploratory Project PI: Huang), NFL (PIs: Huang, Lee), McDonnel Foundation via Brain Trauma Foundation (PI: Ghajar; site PIs: Lee, Huang)
  - Investigator Collaboration: VA San Diego Healthcare System, UCSD.

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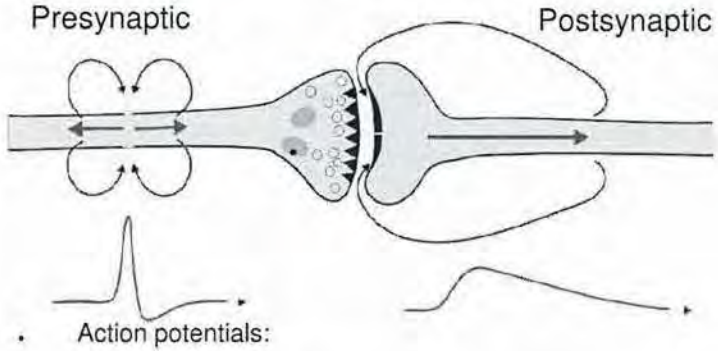
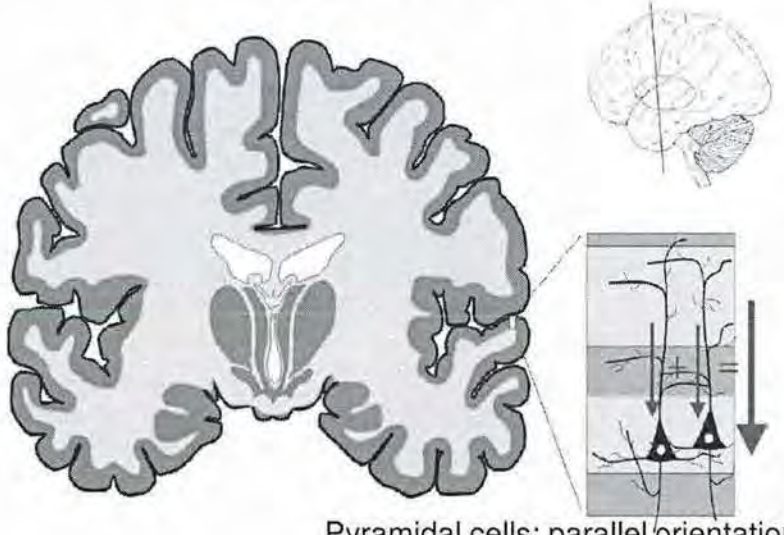
The generation of MEG signal from neuronal current in gray-matter neurons

Neuronal currents in axons and dendrites

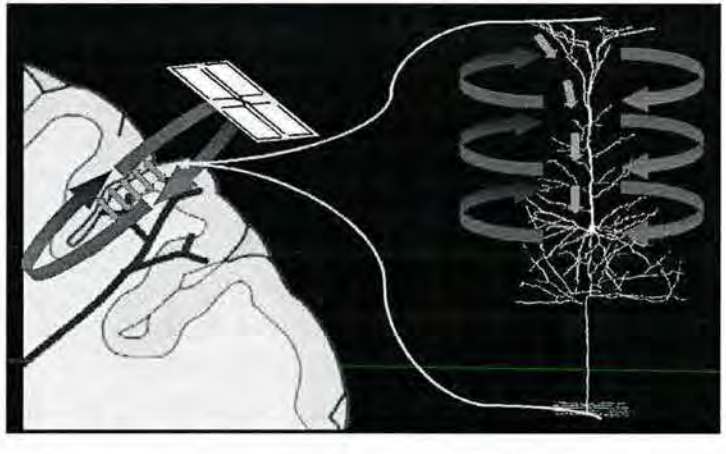


- Action potentials:
  - Fast: no/little temporal summation
  - Cancellation: fields diminish rapidly

Parallel dendrites



Pyramidal cells: parallel orientation
=> spatial summation



First GWAS in Dopamine Beta Hydroxylase confirms strong cis-acting variants and lends support for its role as an intermediate phenotype in post-traumatic stress disorder

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VA CENTER OF EXCELLENCE CESAMH STRESS AND MENTAL HEALTH

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Introduction

Dopamine beta-hydroxylase (DBH) catalyzes formation of norepinephrine. DBH is expressed in noradrenergic nerve terminals of the central and peripheral nervous systems, as well as in chromaffin cells of the adrenal medulla. DBH is present in cerebral spinal fluid and plasma (pDBH) as stable heritable trait. Differences in DBH expression or activity might reflect a **role in the pathogenesis of cardiovascular and neuropsychiatric disorders.** The genetic mechanisms underlying DBH activity and its secretion **have been only partially explained** and no genome wide search has yet been performed.

We investigated

1) if a genome wide search would identify additional variants acting on pDBH.
2) the functionality of promoter variants.
3) the causal relationship between pDBH and PTSD symptoms, using a Mendelian randomization approach.

Methods

Participants: Marine Resiliency Study (MRS) males of European (EA) or Hispanic/Native American (HNA) descent (N=434) (Baker et al. 2012).

DBH measure: Measured in plasma (pDBH) by a modified Nagatsu/Udenfriend spectrophotometric method.

PTSD measure: CAPS re-experiencing symptom score (subscale B).

Genotyping: Illumina HOEE 8V1 array. QCed using standard methods.

Imputations: IMPUTE2 with 1000 Genomes Project reference subjects.

Association between SNPs and pDBH (GWAS):

- Linear regression of pDBH on SNP, with covariates for age and 3 principal components (PCs), for population stratification.
- Separate analysis of EA (N=341) and NA (N=93) ancestry groups, combined in inverse variance weighted meta analysis
- Additional follow up analysis was conditioned on *DBH* promoter SNPs.

In vivo association of promoter variant haplotypes and pDBH enzymatic activity: Performed using linear regression models and ANOVA based on an additive genetic model, with age, cohort, and 3 PCs as covariates.

In vitro functional effects of trait-associated *DBH* promoter variants: *DBH* promoter/reporter haplotypes and additional variants were constructed and co-transfected into pheochromocytoma cells. Analysis of variance (ANOVA) was used to compare luciferase reporter activity between different *DBH* haplotypes.

Mendelian Randomization: Estimates for the effect of pDBH on PTSD symptoms were derived using a control functions approach, where rs1611115 was used as an instrumental variable for pDBH.

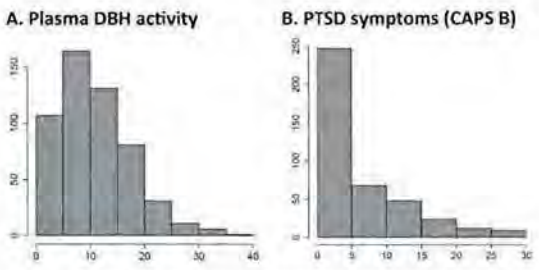

Figure 1: Histograms of phenotypes under investigation.

Results

1. GWAS for plasma DBH in 434 men of European or Hispanic/Native American descent

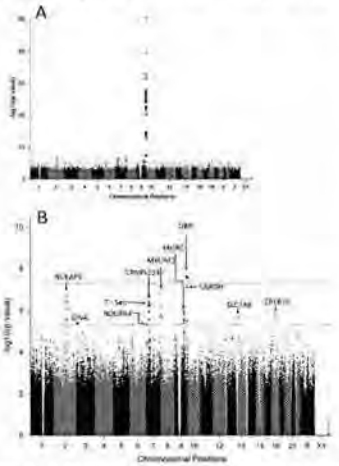

Figure 2: **A.** Manhattan plot of the GWAS for pDBH in EA subjects. **B.** Close up of the Manhattan plot.

		EA GWAS		NA descent GWAS		Meta-analysis	
Gene	SNP	Beta	P	Beta	P	Beta	P
DBH	rs1076150	-0.95	2.7E-32	-0.78	7.7E-06	-0.92	1.4E-44
DBH	rs1989767	0.92	2.3E-26	0.75	1.9E-04	0.90	1.5E-34
DBH	rs1611115	-1.27	7.2E-51	-1.20	4.1E-15	-1.25	4.6E-92
FRRS1	rs7540659	-0.02	0.80	-0.79	3.6E-06	-0.40	0.30
CR617033	rs60678788	0.40	8.1E-05	0.54	0.004	0.44	7.6E-07
NCKAP5	rs459781	0.50	2.2E-07	0.10	0.64	0.43	5.1E-07
KYNU	rs77518496	0.01	0.96	1.25	3.9E-06	0.62	0.33
NBEAL1	rs2351772	0.35	1.7E-04	0.44	0.004	0.37	1.6E-06
BC069004	rs112239400	0.51	7.4E-04	1.08	1.3E-04	0.65	7.7E-07
DIVA	rs13095128	0.67	4.0E-06	0.63	0.08	0.06	0.93
ATXN7	rs3774729	0.00	1	0.87	9.6E-07	0.42	0.33
AK023371	rs56030924	0.02	0.85	0.89	6.4E-07	0.44	0.31
PSMD6	rs831692	0.03	0.73	0.89	6.7E-07	0.45	0.30
PRICKLE2	rs56237630	0.00	0.99	0.94	1.5E-06	0.46	0.33
EPHB1	rs12639432	0.34	2.4E-04	0.55	4.7E-04	0.40	4.2E-07
NDUFA4	rs7779937	-0.92	4.7E-06	0.64	0.33	-0.27	0.73
CR585224	rs13242648	0.55	3.9E-07	0.46	0.05	0.07	0.89
7-Sep	rs12701456	0.55	5.0E-07	0.39	0.10	0.10	0.828
MYOM2	rs13255008	0.50	1.5E-07	0.03	0.88	0.27	0.31
MURC	rs1338730	0.42	1.2E-06	0.40	0.04	0.42	7.4E-08
GRIN3A	rs823919	0.56	6.6E-06	0.28	0.19	0.51	4.0E-06
SARDH	rs7857468	0.54	8.1E-08	0.14	0.55	0.48	1.2E-07
BC032914	rs10795764	0.09	0.27	0.75	2.3E-07	0.41	0.23
IAH1P3	rs820553	-1.21	0.004	-1.26	2.8E-04	-1.24	1.8E-06
DPYSL4	rs112825992	-1.25	0.006	-1.21	2.9E-04	-1.22	2.7E-06
LOC338797	rs4255616	0.39	1.3E-05	0.50	0.00	0.42	4.8E-08
SLC7A8	rs8013529	-0.57	2.4E-06	0.32	0.33	-0.18	0.69
AKAP13	rs12505683	0.01	0.95	-1.20	5.9E-07	-0.58	0.13
QRICH2	rs117713052	1.16	4.1E-05	1.57	0.014	1.23	1.5E-06
PRPSAP1	rs115127145	1.14	4.8E-05	1.57	0.014	1.21	1.7E-06
ZBTB7C	rs7228140	-0.94	1.9E-06	0.44	0.17	-0.27	0.69
Conditional Analysis							
SARDH	rs7857468	0.50	2.4E-11	0.49	0.002	0.50	1.3E-16
SARDH	rs7040170	0.44	7.6E-12	0.46	0.004	0.44	1.3E-14

2. Influence of functional *DBH* variants on plasma DBH activity

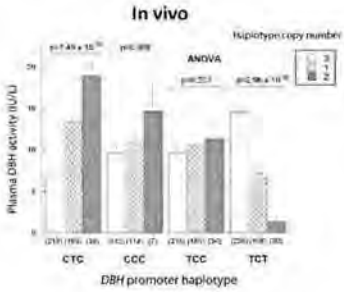

Figure 3: In vivo effects of DBH promoter functional variants on plasma DBH activity.

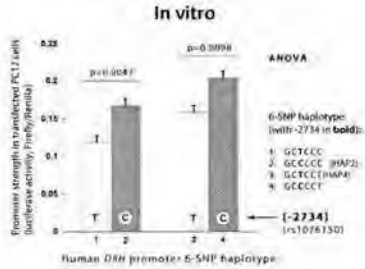

Figure 4: In vitro effects of human DBH promoter variant rs1076150: balanced mutants on two haplotype backgrounds.

3. DBH as a causal predictor of PTSD symptoms

- DBH and PTSD re-experiencing symptoms were found to be **positively associated** (p=0.005)
- rs1611115 was employed as a genetic instrument to test for a **causal effect** of plasma DBH on PTSD symptoms.
- The unconfounded estimate of the association of pDBH and re-experiencing symptoms **was significant** (beta=0.26, p = 0.002)

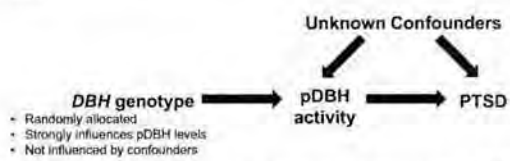

Figure 5: *DBH* used to produce an unconfounded estimate of the causal effect pDBH on PTSD.

Conclusions

1) First GWAS of plasma DBH has identified:

- The *DBH* gene as the principal locus determining pDBH levels (R^2 = 57%) in both EA and NA populations.
- Two novel loci, *SARDH* and *LOC338797*

2) SNP rs1076150 has a functional effect on transcription in vivo and in vitro.

3) We found evidence of causal association between plasma DBH and PTSD symptoms.

In perspective, the characterization of DBH activity and its underlying genetic regulation has positioned us uniquely for future studies of 'intermediate phenotypes', potentially leading to discovery of causal variants in complex genetic traits and disorders such as found in the psychiatric and cardiovascular fields.

Contents appear in print: The catecholamine biosynthetic enzyme dopamine β-hydroxylase (DBH): first genome-wide search positions trait-determining variants acting additively in the proximal promoter *Hum. Mol. Genet. ddu332 first published online June 30, 2014 doi:10.1093/hmg/ddu332*

Impact of Childhood Maltreatment on Physical Health-Related Quality of Life in U.S. Active Duty Servicemen and Veterans

Laura Aversa,Ph.D.[1]; Jennifer Lemmer, Ph.D., M.P.H.[2]; Sarah Nunnink, Ph.D.[1,3], Robert McLay, M.D., Ph.D.[4] & Dewleen G. Baker, M.D.[1,3,6] [1] VA San Diego Healthcare System [2] Central Texas VA Healthcare System
[3] Department of Psychiatry, University of California, San Diego [4] Naval Medical Center, San Diego [5] VISN 22 Center of Excellence for Stress and Mental Health

Background

Childhood maltreatment, depression and PTSD are related to poor physical health outcomes and health-related quality of life (HRQoL).

Higher rates of history of childhood maltreatment have been documented in veterans and active duty service members of the US Armed Forces compared to the general population, and has been shown to be a direct risk factor in the development of combat-related PTSD.

Childhood maltreatment also increases the risk of development of mental health problems such as depression, poor perceptions of health, and poor health status.

Our study aimed to further understand the effect of childhood maltreatment, PTSD, and depression on physical HRQoL in an all-male sample of US military veterans and active duty servicemen.

Specifically, our study aimed to examine whether PTSD or depressive symptoms mediate the relationship between childhood maltreatment and physical HRQoL in a male military sample, as PTSD was found to mediate the relationship between childhood maltreatment and physical HRQoL in a sample of female veterans .

Methods

Participants were 249 male OEF/OIF active duty service members and combat veterans who volunteered to participate in a research study examining genetic risk factors associated with PTSD.

Participants were recruited at the VA San Diego and Naval Medical Center through clinician referrals or self-referral. The sample included both individuals with diagnostic levels of depression and PTSD and those with sub-threshold or absence of symptoms.

Inclusion criteria: Moderate level of combat exposure, having returned from deployment for at least 6 months.

Exclusion criteria: Current alcohol or drug dependence and a self-reported history of a pre-deployment Axis I disorder.

Participants completed self-report questionnaires including the Combat Exposure Scale (CES), the Childhood Trauma Questionnaire (CTQ), and the Medical Outcomes Short-Form 36 (SF-36).

Participants also completed a clinical interview and were administered the Clinician-Administered PTSD Scale for DSM-IV (CAPS) and the Hamilton Depression Rating Scale (HAM-D).

Table 1. Sample Characteristics

Demographics and Predictor Variables	N=249	SF-36 Sample Means (N=249)	M (SD)
Age M (SD)	29.0 (7.1)	Physical Component Score	51.7 (9.7)
Active Duty N, %	112 (45.0)	Physical Functioning	82.9 (23.3)
Caucasian N, %	192 (77.0)	Role-Physical	71.4 (39.1)
Combat Exposure M (SD)	26.4 (9.6)	Bodily Pain	63.0 (24.9)
CTQ Total Score M (SD)	40.7 (14.7)	General Health	68.2 (21.6)
CAPS Total Score M (SD)	53.9 (32.2)	Vitality	45.9 (22.4)
HAM-D Total Score M (SD)	10.0 (7.3)	Social Functioning	62.9 (32.0)

Analyses

Multiple regression was employed to estimate an omnibus model of the relation of PTSD symptoms, depressive symptoms, and childhood maltreatment to the Physical Component Summary (PCS) of the SF-36.

Mediation was tested using the criteria that the independent variable must predict the mediator, the mediator must predict the dependent variable, and the independent variable must predict the dependent variable in the absence of the mediator (Baron & Kenny, 1986). A Sobel test was conducted to determine statistical significance of the mediation effect.

Results

Sample characteristics are reported in Table 1. Both PTSD and depression mediated the relationship between childhood maltreatment and the overall physical HRQoL summary score (Sobel t=-2.14, p=.03 for PTSD; t=-2.14, p=.03 for depression).

Table 2 reports the results of omnibus regression. Depression explained a significant proportion of the variance in HRQoL, 10% of the unique variance.

Table 2. Multiple Regression Predicting Physical Composite Summary

Overall Model $R^2 = 0.14$ *F for* $\Delta R^2 = 8.79$ $p<.001$

Predictor	B	SE	t
Constant	57.20	2.33	---
Duty Status	1.72	1.24	1.38
Combat Exposure (CES)	-0.01	0.08	-0.15
Childhood Maltreatment (CTQ)	-.006	0.04	-1.46
PTSD symptoms (CAPS	.016	.003	0.50
Depressive symptoms (HAM-D)	-0.45	0.13	-3.50 (p<.001)

Conclusion

PTSD and depression both mediated the relationship between overall physical HRQoL and childhood maltreatment.

In a sample of all-male veterans and active duty service members, we found similar impairment in overall physical HRQoL as has been demonstrated in all-female and community samples.

Our study lends further support to previously published findings that identified depression as a factor with a large effect on physical HRQoL. Previous studies have examinec PTSD as a mediator between childhood maltreatment and physical HRQoL, but there has been less focus on depression as a mediator.

The meditational relationship is important and may lend further support to neurobiological research that early adverse experiences can sensitize the central nervous system and lead to increased risk for anxiety and depressive disorders.

Acknowledgement: Funding was provided by VA Clinical Research and Development (VA CSR&D)

Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in a Sample of Active Duty Marines

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[1]Department of Psychiatry, University of California San Diego; [2] Center for Excellence in Stress and Mental Health and [3]Mental Illness Research, Education and Clinical Center, VA San Diego Healthcare System

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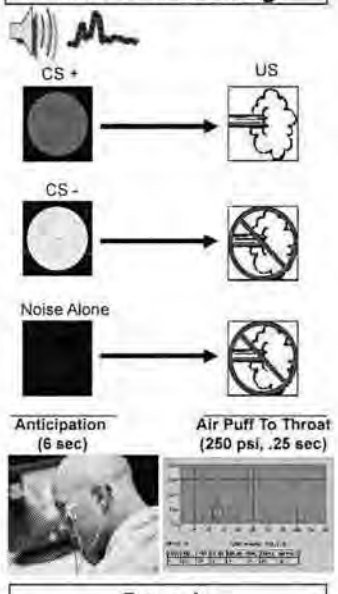
Background

- The Marine Resiliency Study II (Oct 2011-Oct 2013) Neurocognition project is a longitudinal investigation of neurocognitive predictors of risk and resilience to trauma effects in Marines deployed to Afghanistan.
- As part of this investigation, >1200 Marines and Navy corpsmen underwent a fear conditioning and extinction paradigm and psychiatric symptom assessment prior to OEF deployment and 3-6 mo after their return.
- The current analysis tested the hypotheses that fear learning and extinction abnormalities may be markers of distinct anxiety symptom classes in the Negative Valence Domain.
- To test this hypotheses we examined FPS performance at pre-deployment in subjects grouped by primary symptom class.

Methods

- Subjects underwent fear conditioning and extinction procedure prior to deployment along with assessment of psychiatric symptoms.
- 2 Phases:
  - 8 Fear Acquisition Trials
  - 16 Fear Extinction Trials
- Fear responses assessed via potentiated startle and post-phase self-reported anxiety.
- Symptom groupings:
  - PTSD (CAPS, 1B,2C,2D symptoms with intensity >2; Stein et al., 1997)
  - Anxiety (BAI > 15)
  - Depression (BDI-2 > 19)

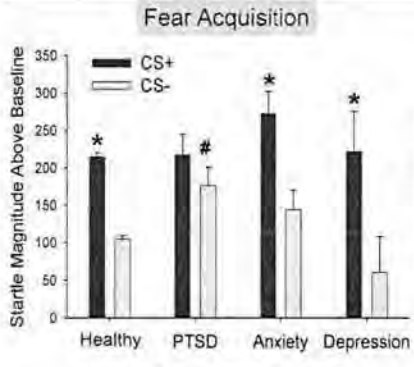
Fear Conditioning

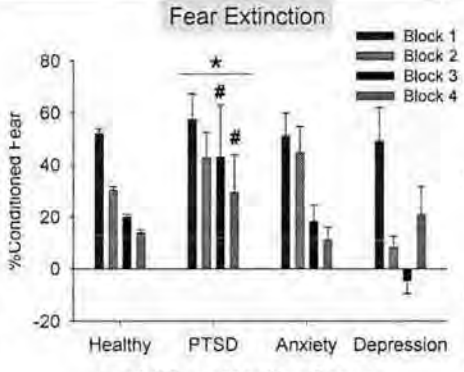


Sample

Symptom Group	N
Healthy	923
Moderate-Severe PTSD	42
Moderate – Severe Anxiety	37
Moderate – Severe Depression	12

Potentiated Startle Results

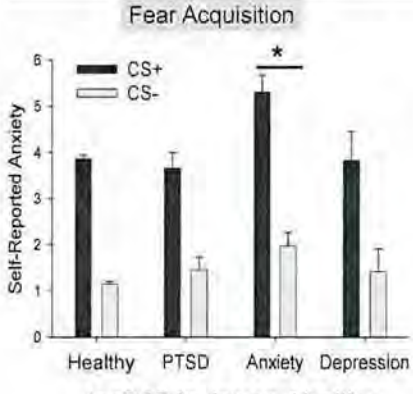


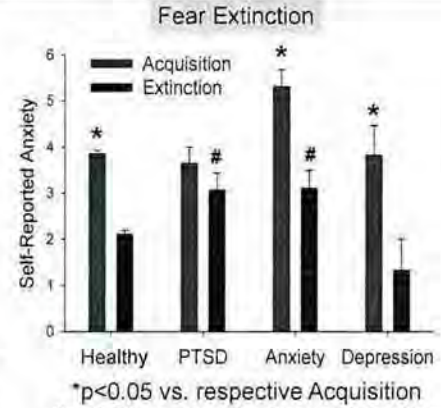


*p<0.05 vs. respective CS-
#p<0.05 vs. Healthy Group CS-

*p<0.05 vs. Healthy Group
#p<0.05 vs. Healthy Group respective block

Post-Phase Self-Reported Anxiety Results





*p<0.05 Anxiety vs. Healthy

*p<0.05 vs. respective Acquisition
#p<0.05 vs. Healthy Group Extinction

Data Analysis

- Subjects classified into multiple pathology categories (comorbidity) were removed to achieve "pure" effects of pathology on conditioning measures. (N=35).
- EMG response during Noise Alone trials was subtracted from CS responses across phase to control for baseline startle/habituation.
- Startle trials grouping:
  - Acquisition = last 4 trials of each CS
  - Extinction Blocks= Mean 4 CS+ trials calculated as percentage of max CS+ responding during Acquisition
- Post-Phase anxiety also evaluated .

Results & Summary

- Distinct patterns of FPS learning and extinction emerged differentiating the PTSD and Anxiety symptom classes from both Healthy and Depression classes.
- Only the PTSD group displayed deficient safety signal discrimination and reduced extinction learning.
- The Anxiety group showed normal safety signal discrimination and extinction, but exhibited increased baseline startle reactivity (data not shown) and higher self reported anxiety to both cues.
- The Depression group was not different from the Healthy group (though small N).
- Deficient safety signal and extinction learning are relatively specific marker s of PTSD symptoms and these effects are not confounded by increased overall fear responses.
- Ongoing longitudinal analyses will examine whether these markers are vulnerability traits or occur only after trauma in this prospective sample.

For details see: Acheson et al. Conditioned Fear and Extinction Learning Performance and its Association with Psychiatric Symptoms in Active Duty Marines. *Psychoneuroendocrinology*, in press.

Identifying Extinction Learning Trajectories and their Association with Psychiatric Symptoms in a Sample of Active Duty Marines

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- Center for Excellence in Stress and Mental Health, VA San Diego Healthcare System

Introduction

- Posttraumatic Stress Disorder (PTSD) is a heterogeneous psychiatric condition

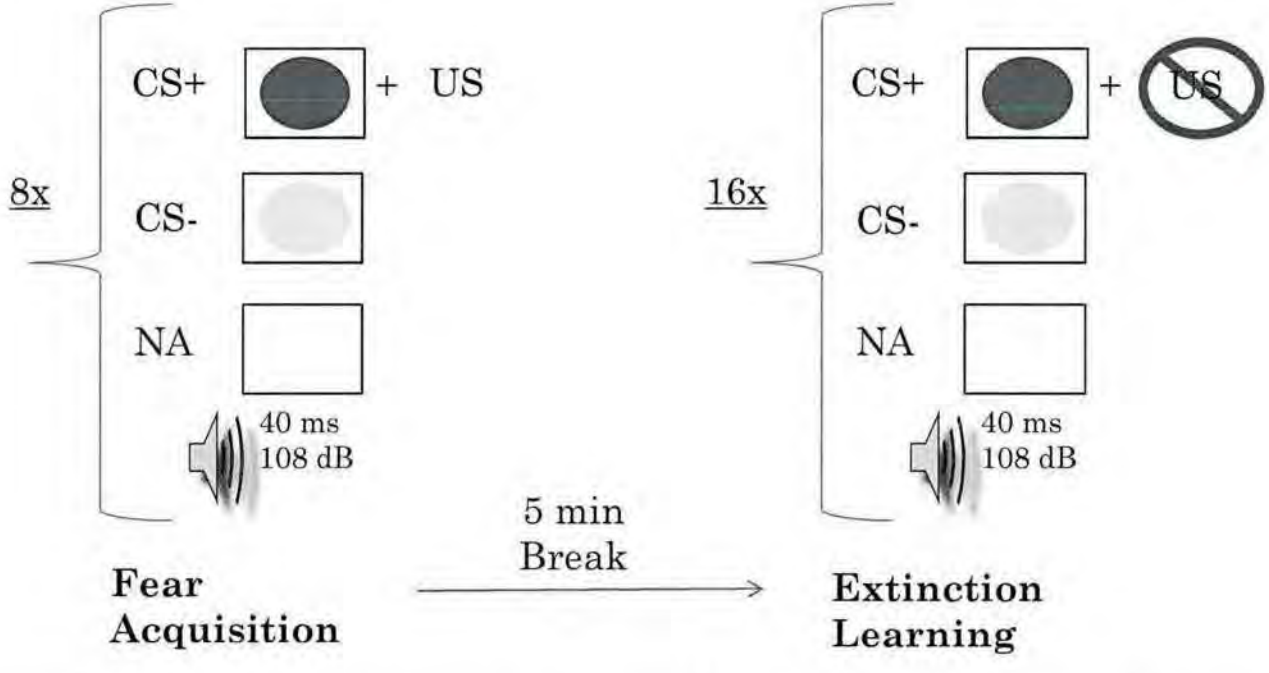
- Current diagnosis of PTSD based on symptoms, rather than specific pattern of biological pathologies

- Critically important to identify biomarkers to aid in:
  1. Understanding etiological pathways
  2. Classification of disorder
  3. Treatment
  4. Prevention

Marine Resilience Study – 2

- Large study of infantry Marines and Navy corpsmen being deployed to Afghanistan aimed at IDing biomarkers of risk/resilience to stress injury
- Pre-deployment time point: N = 1012
- Pertinent Measures:
  - Fear extinction
  - Trauma Load: Life Events Checklist (LEC)
  - Depression: Beck Depression Inventory (BDI2)
  - Anxiety: Beck Anxiety Inventory (BAI)
  - PTSD: Clinician Administered PTSD Scale (CAPS)

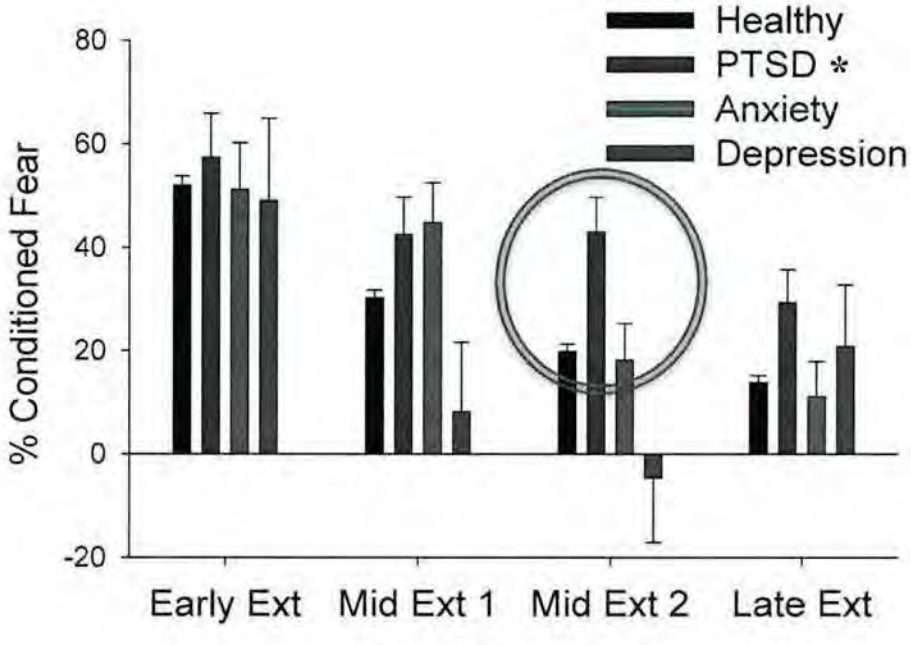
Fear Potentiated Startle Conditioning and Extinction



Response = [Startle in presence of cue(CS)] – [Startle in absence of cue (NA)]

Previous analysis of extinction learning in this sample

- Creation of "index score" based on percent of maximum acquisition startle retained
- 42 Marines with elevated PTSD symptoms showed higher index score across extinction
- Method limited to association with categorical symptom groups at cross-section of extinction
- Need method to associate the full range of symptom severity with individual extinction learning *process*



* = $p<.05$ vs. Healthy

Acheson et al. (2015) *Psychoneuroendocrinology*

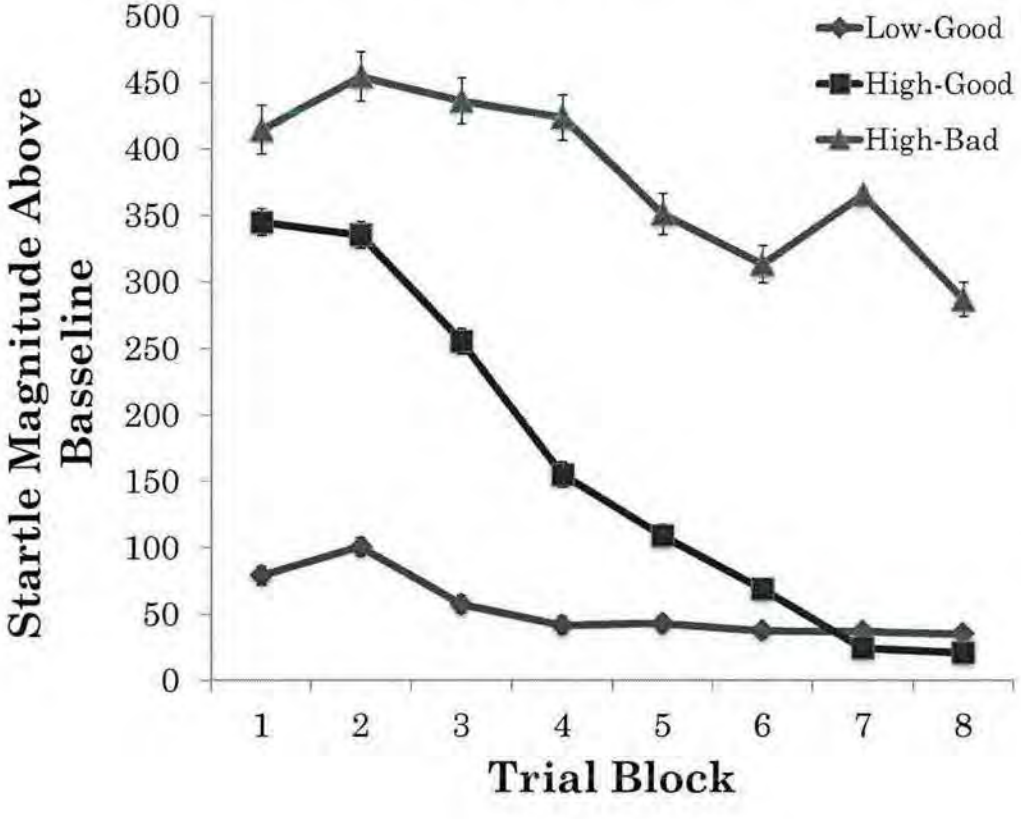
Aims of Current Analysis

- Employ latent class mixture modeling (LCMM) to define groups with distinct extinction learning trajectories
  - Examination of individual learning *process* across phase
  - Non-linear learning curve

- Assess associations between group membership and *current* psychiatric symptoms

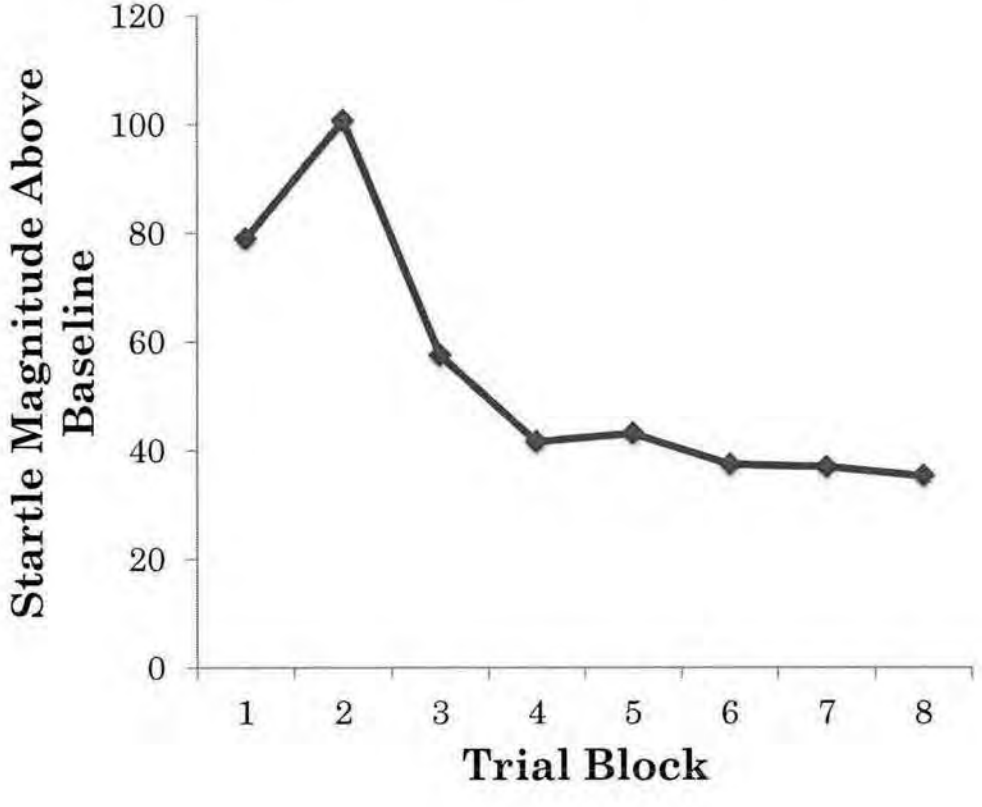
- Hypotheses:
  1. Identify a group characterized by poor extinction learning across the phase
  2. This group will be associated with a range symptoms to a greater degree than group characterized by good extinction learning

Identification of Latent Extinction Trajectory Classes



	N	%	Class Prob.
High - Bad	90	9%	.87
High - Good	296	29%	.75
Low - Good	626	62%	.83
Total	1012		

Identification of Latent Fear Extinction Trajectory Classes



Class Characterization

	Age	Months in Military	Previous OIF/OEF Deployment
Low - Good	22.19 (3.08)	32.50 (29.38)	16%
High - Good	22.16 (2.59)	30.69 (25.15)	14%
High - Bad	22.7 (3.54)	32.48 (28.92)	17%
Total	22.23 (2.99)	31.98 (28.19)	15%

No differences by:
Racial Background
Ethnicity
Educational Level
Marital Status

Class Characterization

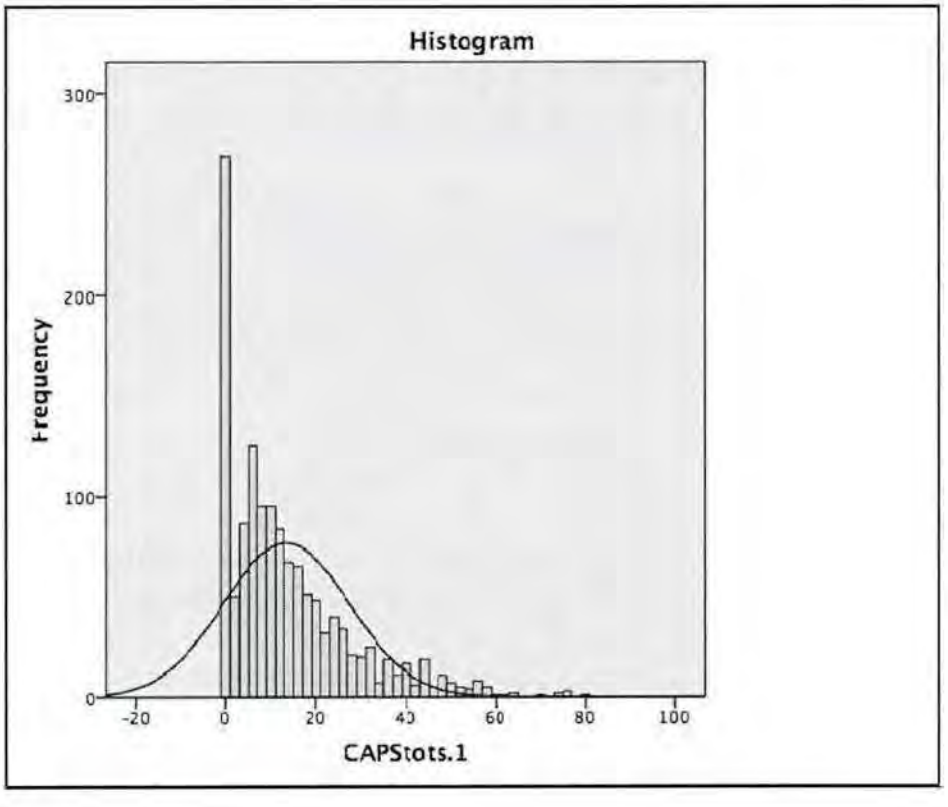
	CAPS Total	BAI Total	BDI Total	LEC Events
Low - Good	12.69 (13.22)	3.95 (5.69)	5.28 (6.33)	4.39 (2.88)
High - Good	12.58 (13.67)	**4.73#** **(6.67)**	5.34 (6.42)	4.33 (3.00)
High - Bad	13.62 (13.26)	**5.74*** **(6.22)**	5.00 (5.20)	4.69 (3.16)
Total	12.74 (13.34)	4.33 (6.05)	5.28 (6.33)	4.39 (2.94)

*$p < .05$; #$p < .1$

Class Characterization

	Assault Any	Past Sexual Assault	Past Combat Experience
Low - Good	65%	2.2%	13%
High - Good	63%	1%	14%
High - Bad	72%	2.2%	15%
Total	65%	2%	13.4%

Problem with Non-Normal DVs



Zero Inflated Negative Binomial Regression

Two Models

- Zero model: Logistic regression modeling probability symptom-free
- Count model: Negative binomial regression modeling predicted symptom severity

Models

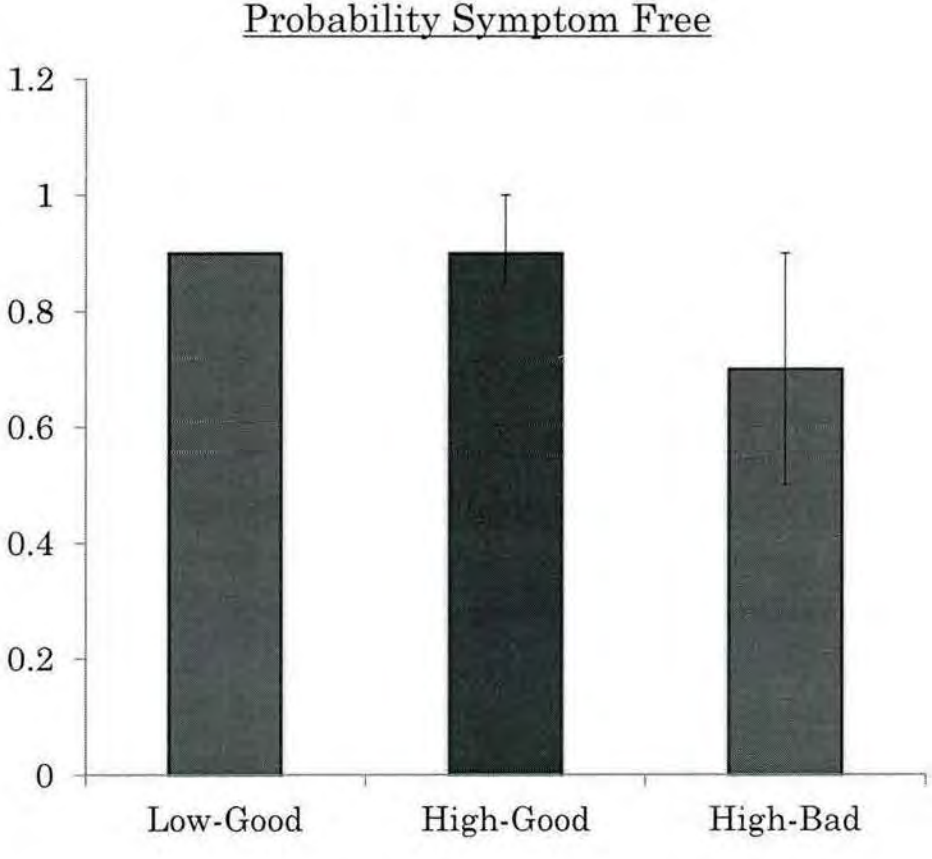
1. DV: CAPS and BAI
   - Predictors: LEC + BDI + Class

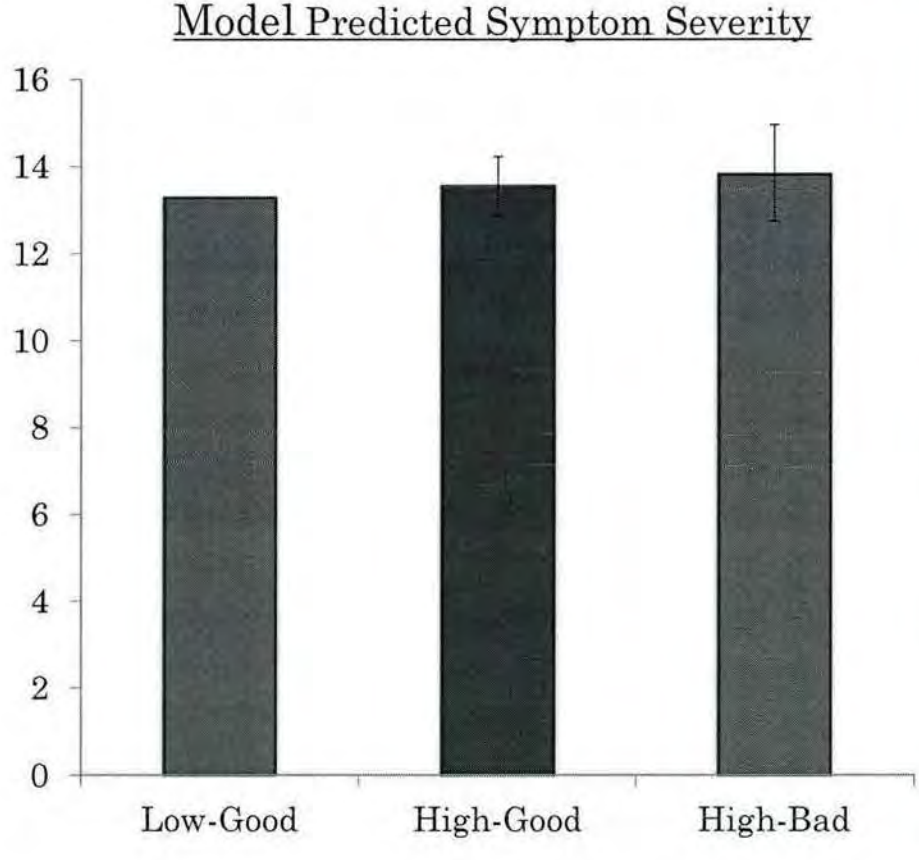
2. DV: BDI
   - Predictors: LEC + BAI + Class

"Low-good" class as referent in both models

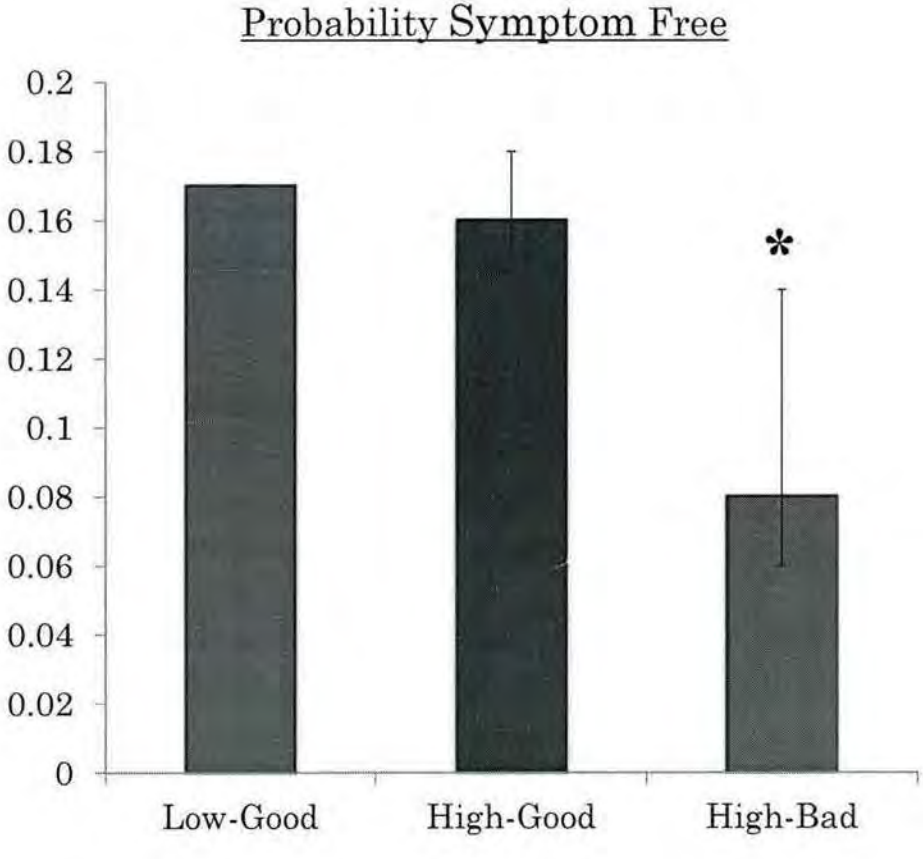
Predicted probability symptom free and predicted symptom severity by class given average LEC and BDI/BAI.

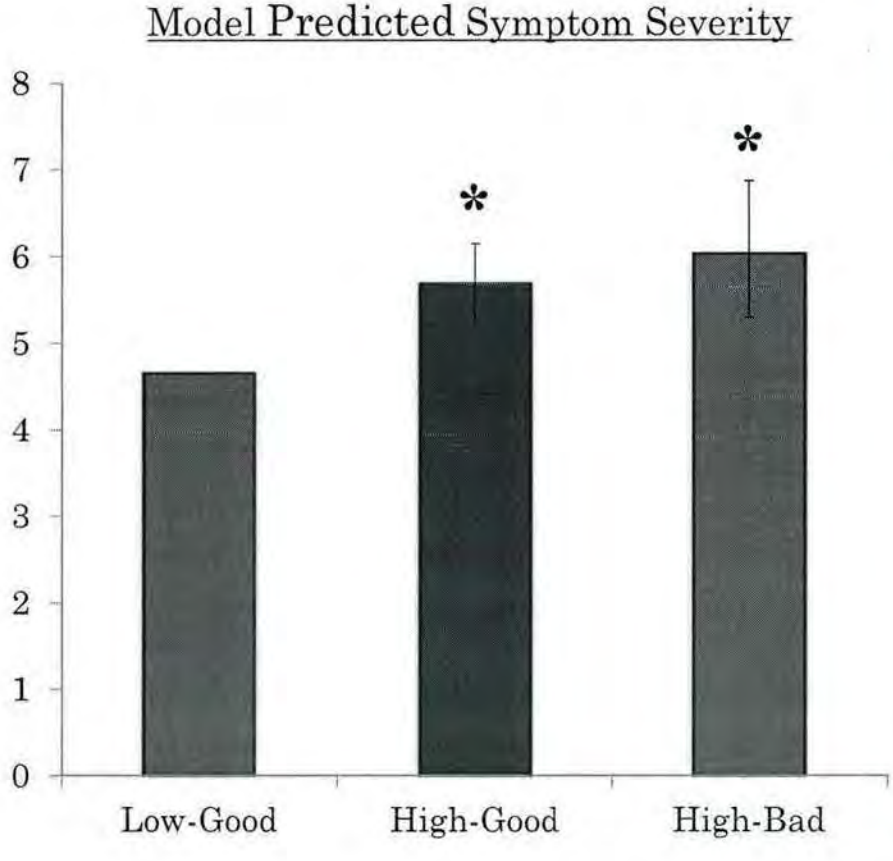
CAPS Total Score



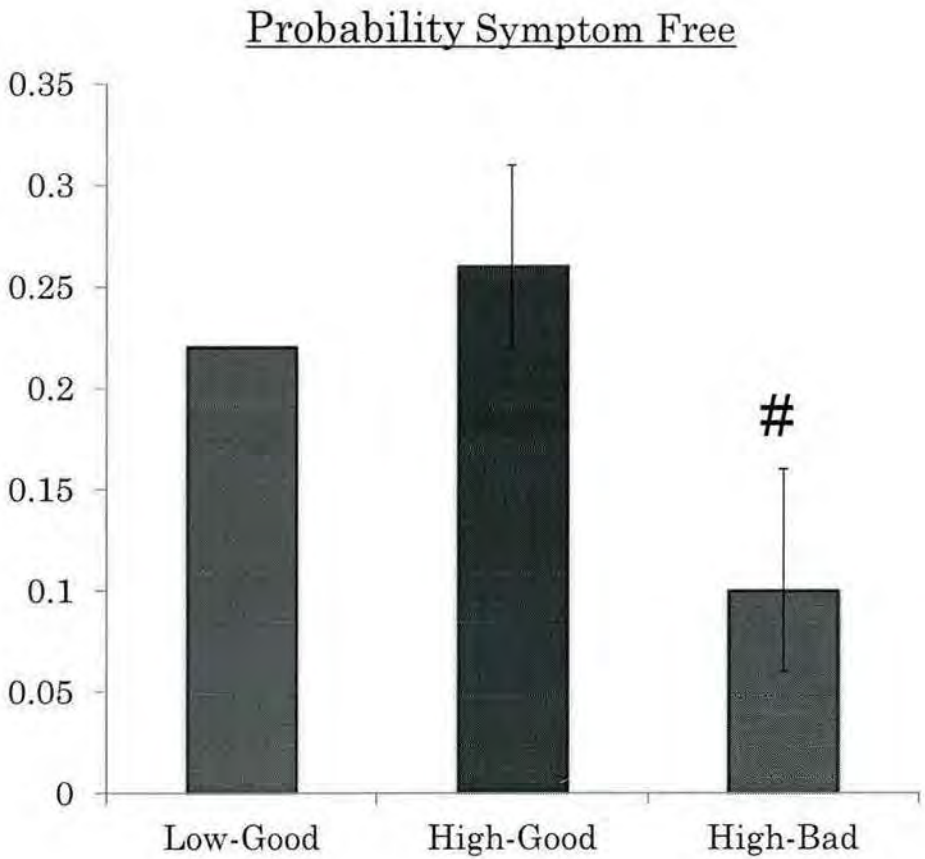


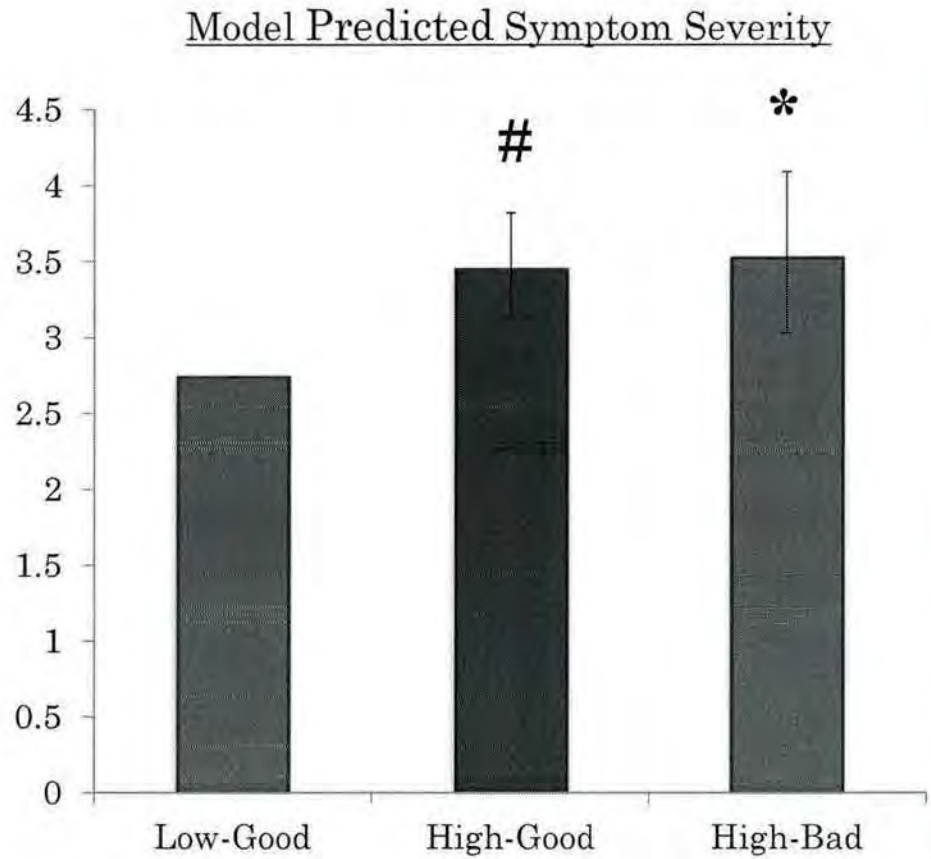
Beck Anxiety Inventory (BAI)



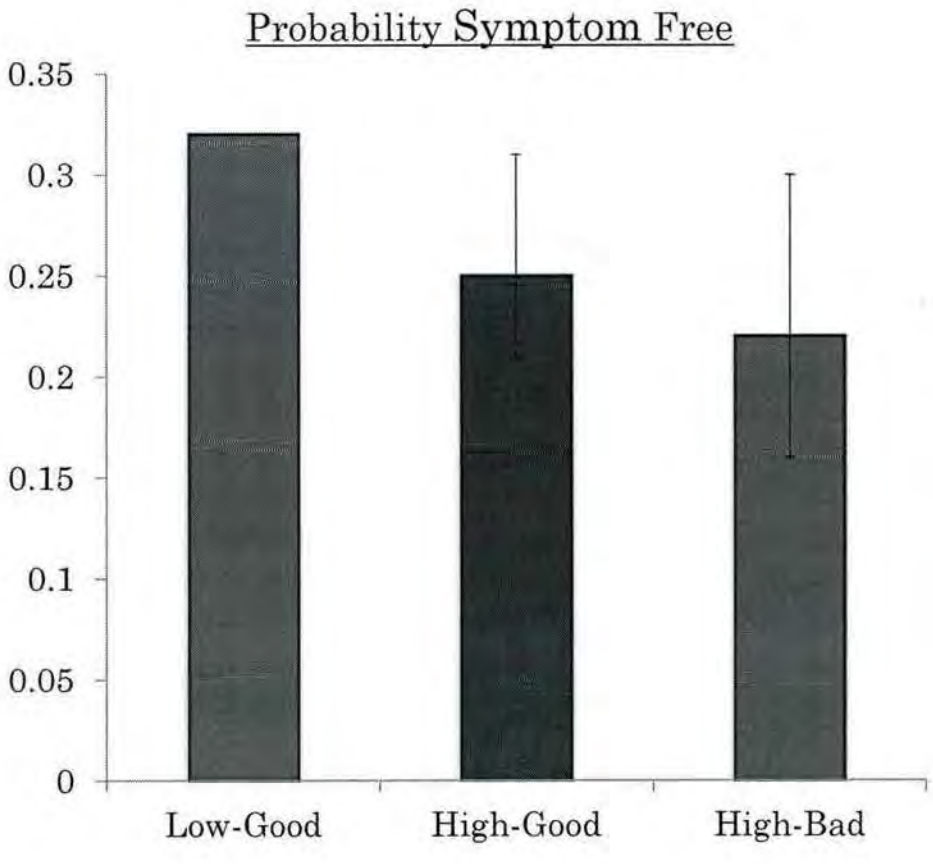


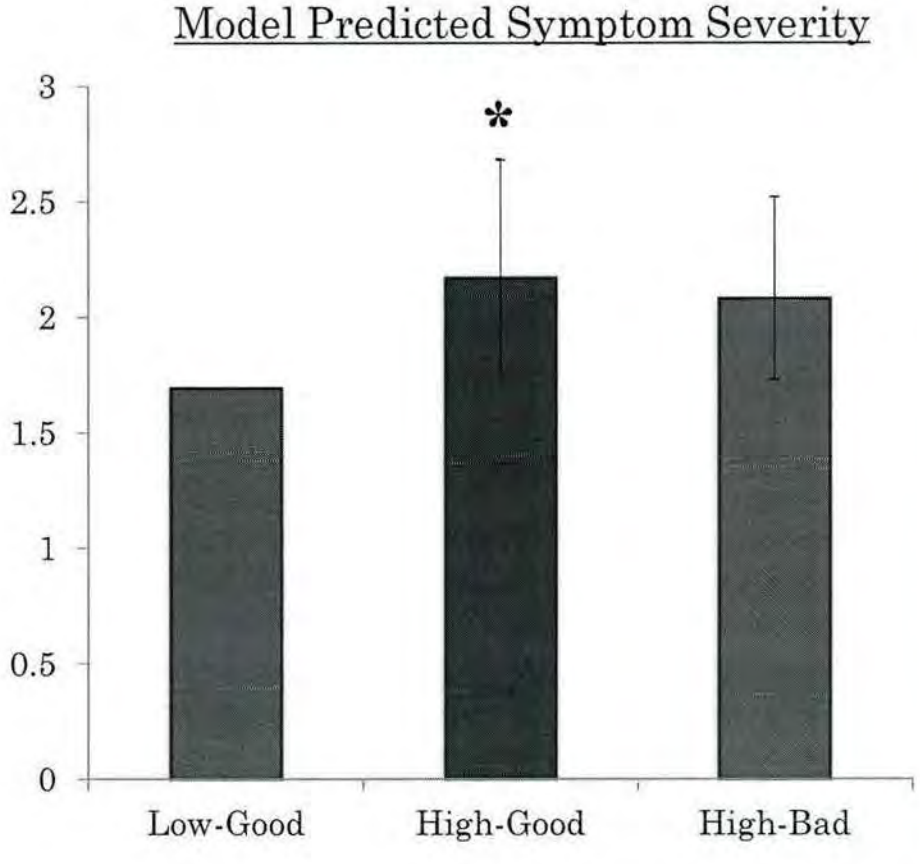
BAI – Somatic Symptoms





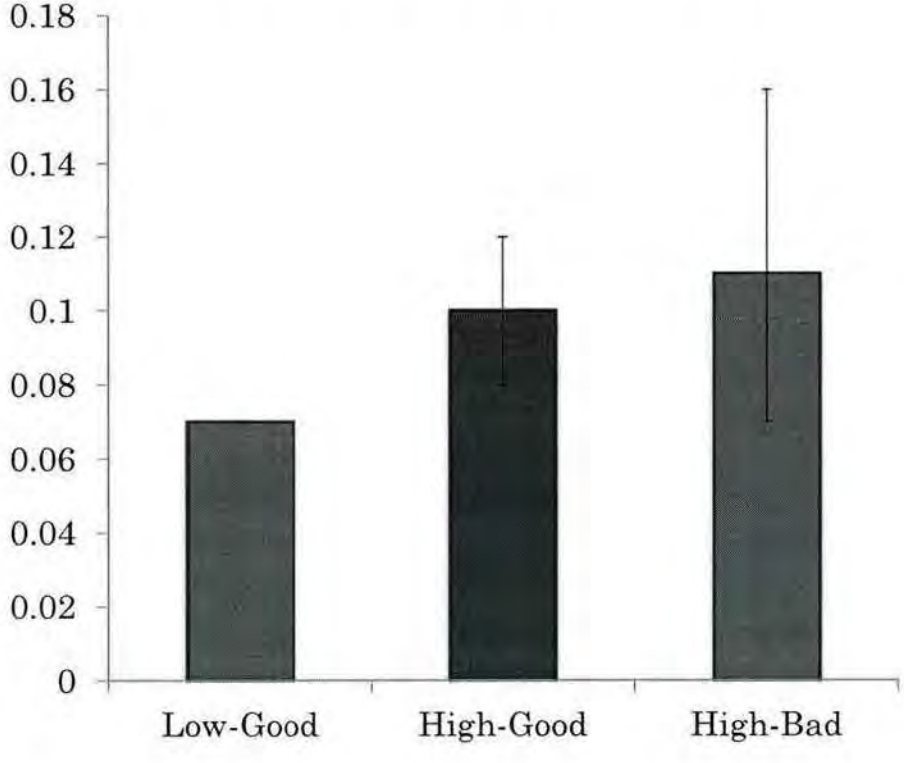
BAI – Cognitive Symptoms



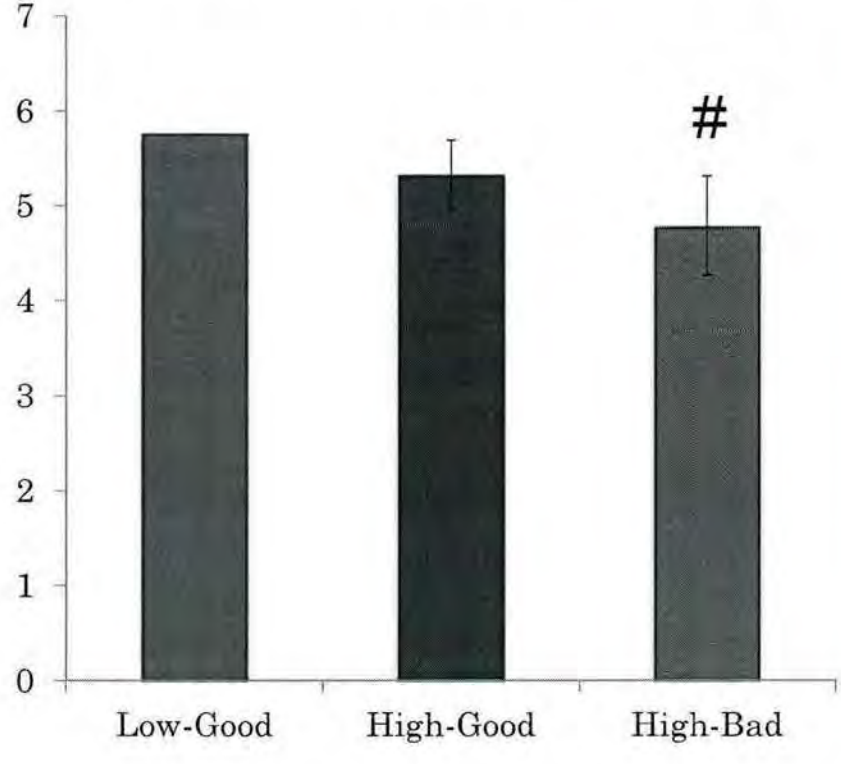


Beck Depression Inventory

Probability Symptom Free



Model Predicted Symptom Severity



Summary

- Identified three distinct trajectory classes for extinction learning process
  - No class differences on demographic variables or "trauma load"
- Class membership, while not associated with CAPS, was with higher severity and incidence of general anxiety symptoms
  - Effect more clear for somatic symptoms
  - Opposite pattern emerged for depression, though non-significant
- Class membership may be associated with syndrome characterized by high physical anxiety symptoms and lower depressive symptoms
  - Kuhn et al. (2014): Enhanced fear extinction in major depression
- Study is limited by homogeneity of sample.
  - Young, healthy, mostly Caucasian, all male
  - Repeat in more heterogeneous sample with higher illness frequency

Future Directions

- Replicate current results with fear extinction performance post-deployment
  - Assess associations following recent stressor
- Does pre-deployment group membership predict development of symptoms post-deployment?
- Does class membership amongst those meeting disorder criteria predict response to exposure-based therapy?

Acknowledgements

MRS Investigators

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Andrew De La Rosa

Richard "Pete" Sharp

Adam Maihoffer

Entire MRS Team

UCSD

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Stress and Mental Health

Acoustic Startle Threshold: Predictor of Psychiatric Symptoms Pre- and Post-deployment

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INTRODUCTION

- Identification of biomarkers of posttraumatic stress disorder (PTSD) is essential for developing more effective PTSD treatment and prevention strategies.
- Exaggerated startle is a symptom of PTSD, yet evidence for altered startle in PTSD is mixed[1].
- Most research on startle in PTSD has focused on startle response magnitude, but "increased startle" in PTSD patients may refer to elevated probability a startle response under sub-threshold conditions[2].
- Prospective studies are necessary to distinguish elevated startle as a consequence of PTSD (state) versus a pre-existing risk (trait) marker.

METHODS

PARTICIPANTS

•2600 Marines and Navy Corpsmen, participating in Marine Resilience Study[3]

MEASURES:

- *Startle threshold test*: startle probes presented over 80-114db levels (pre-deployment)
- *Clinical symptom scales* (administered pre-deployment, 3 and 6 months post-deployment):
  - •Clinician Administered PTSD Scale (CAPS)
  - •Beck Anxiety Inventory (BAI)

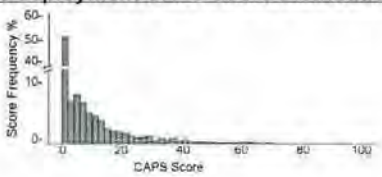
ANALYSIS

•Latent class mixture growth analysis modeled individual startle performance into one of three class-specific trajectories: "high," "moderate," and "low" startle threshold.

•Given skewed psychiatric symptom distribution and excess of zero-scores, zero-inflated negative binomial regression (ZINBR) used to assess relationships between startle threshold class with pre-deployment and 6-month post-deployment clinical symptoms.

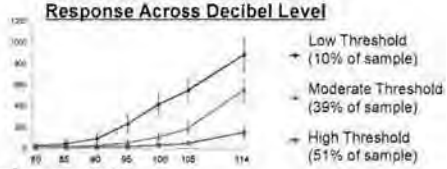
- Other predictors in model included race, battalion, hearing, and previous deployment
- Moderate threshold class was reference group
- ZINBR modeled outcomes via both:
  - •Logistic regression predicting probability of a zero score (absence of symptoms)
  - •Negative binomial regression predicting total symptom score

Pre-deployment CAPS Score Distribution



RESULTS

Mean Startle Threshold Class Response Across Decibel Level



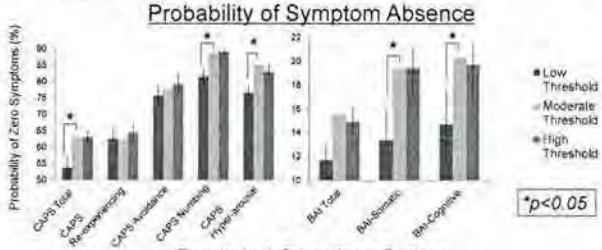
Demographics Across Startle Threshold Classes

	High Threshold (n=1322)	Moderate Threshold (n=1004)	Low Threshold (n=266)
Age	22.69 (3.62)	22.88 (3.42)	22.93 (3.20)
Race*, %			
Caucasian	55.3	68.9	66.0
African American	8.5	4.2	2.6
Hispanic/Native American	18.2	17.0	19.2
Asian/Other	17.9	9.9	10.2
Any previous deployment*	49.5%	51.7%	58.6%

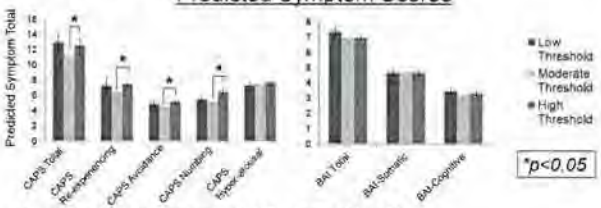
** Significant group differences at p<0.05*

Pre-deployment[a]

Probability of Symptom Absence

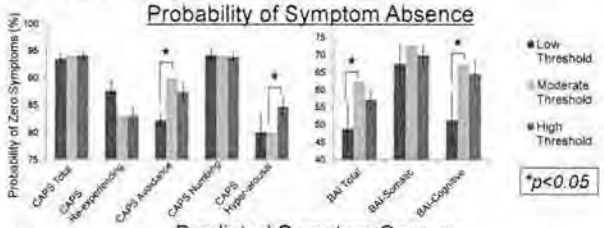


Predicted Symptom Scores

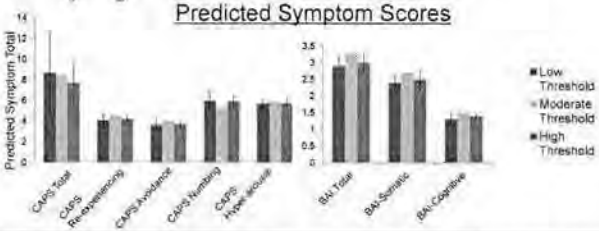


Post-deployment (6 months)[b]

Probability of Symptom Absence



Predicted Symptom Scores



a Pre-deployment estimates for subject in battalion 1, white, never before deployed, with average hearing
b Six month post-deployment estimates for subject in battalion 1, white, never before deployed, with average hearing, and with zero scores on measures at pre-deployment and 3-months post-deployment

DISCUSSION

- Relative to moderate startle threshold class:
  - Low startle threshold → decreased likelihood of being symptom free at pre-deployment, and increased risk for PTSD-related avoidance and anxiety symptoms post-deployment
  - High startle threshold → more severe PTSD symptoms *except* hyperarousal pre-deployment, and reduced risk for hyperarousal symptoms post-deployment
- Moderate startle threshold class (40% of sample) may be marker of both low symptoms (state) and increased resilience to developing PTSD symptoms (trait).
- Individuals with most severe trauma histories may show blunted startle[4] (among subjects meeting lenient pre-deployment diagnostic criteria for PTSD, greater history of childhood trauma for those with high startle threshold).
- Potential biological mechanisms:
  - Differing thresholds for "high stress" activation of periaqueductal grey, which inhibits startle responding in favor of other defensive behaviors[5]
  - Inverted-U shaped dose response function of cortisol on startle reactivity may suggest altered HPA axis across classes, analysis of CORT levels is ongoing.
- Limitations:
  - Limited generalizability of sample: male, young, generally healthy, highly screened military population
  - Few subjects met full diagnostic criteria for PTSD at pre-deployment (3.7%) or post-deployment (5.4%)

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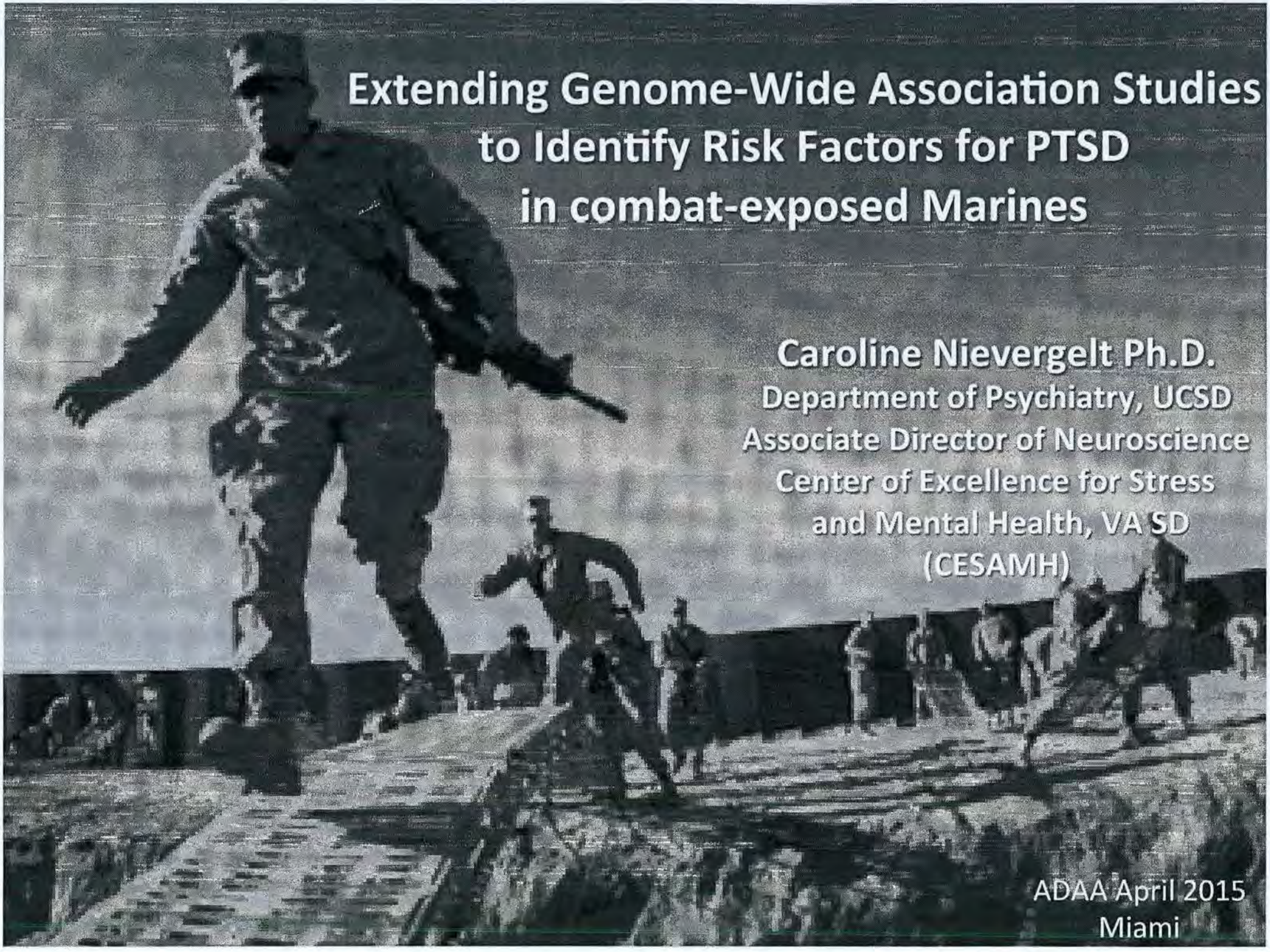

Extending Genome-Wide Association Studies to Identify Risk Factors for PTSD in combat-exposed Marines

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Associate Director of Neuroscience
Center of Excellence for Stress
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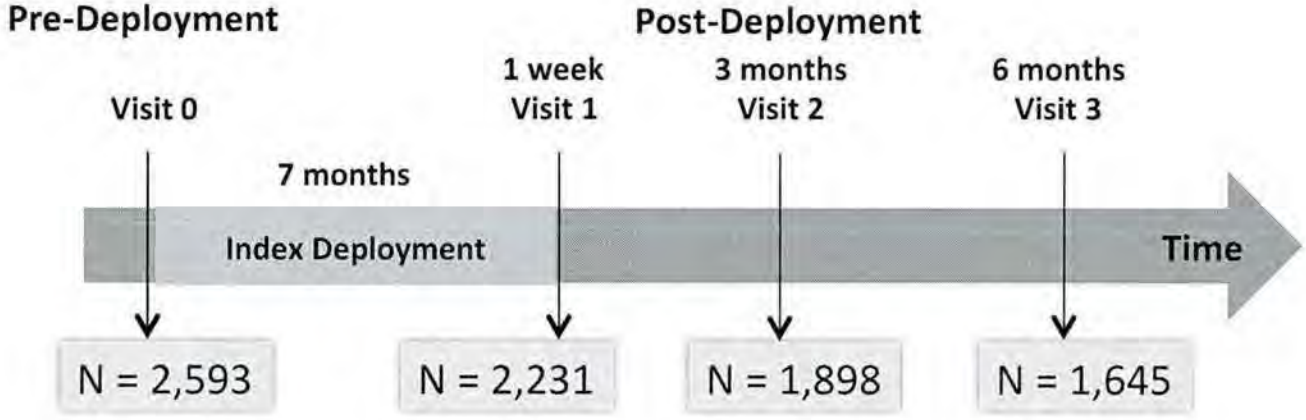
(CESAMH)

ADAA April 2015
Miami

Marine Resiliency Study (MRS)

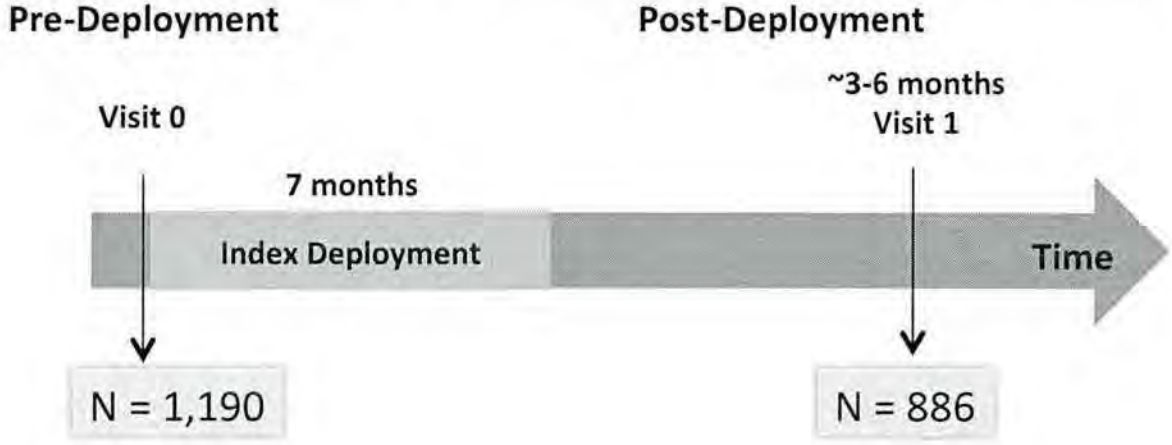
Goal: Predictors of Risk and Resilience for Posttraumatic Stress Disorder

MRS-I: 2008 – 2011 (PIs: Baker, Nash, Litz) Cohorts 1-4



- Prospective study
- Deployment to Iraq or Afghanistan
- Longitudinal follow-up

MRS-II: 2011 – 2013 (PIs: Baker, Risbrough, Geyer) Cohorts 11-12



Timeline and Enrollment

MRS Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews

Historical
- Self-report questionnaires

Neuropsychological
- Attention, Memory, Executive Function, Reasoning, Social Cognition

MRS Secure database

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

Biological assessments

Biomarkers
- NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Metabolomics (subset)

Telomere length (subset)

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- Startle threshold and habituation, fear conditioning and extinction, heart rate variability

Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Genomics

- **GWAS (complete data)**
- **Methylome (subset, pre-post)**
- **Transcriptome (subset, pre-post)**

PTSD diagnosis using CAPS

• **Criterion A.** The person has been exposed to a traumatic event in which both of the following were present:
(1) the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others
(2) the person's response involved intense fear, helplessness, or horror (not for military cohorts)

• **Symptom clusters:**
B: Reexperiencing (B1-5)
C: Avoidance / Numbing (C1-7)
D: Hyperarousal (D1-5)

• **F1/I2 rule: frequency of 1 and intensity of 2** **(range: 0-4 for both)**
experienced it at least once or twice during the last month
distress/discomfort: moderate, distress clearly present but still manageable, some disruption of activities

• **DSM-IV PTSD diagnosis:**
Full: 1B, 3C, 2D ← PTSD dx
Partial stringent: 1B, 2C, 2D
Partial lenient: 1B, 3C OR 2D ← Partial PTSD dx

Broad definition of PTSD

• **CAPS summary score:**
Sum of all frequencies and intensities of the 17 questions (range: 0 – 136)

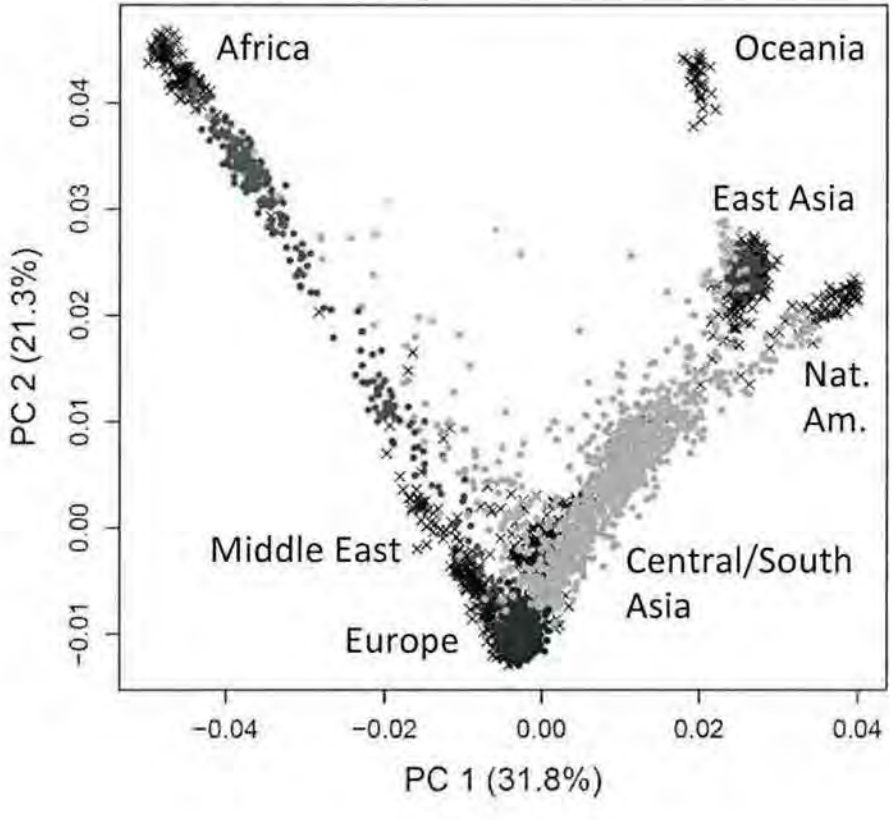
Clinician Administered PTSD Scale (CAPS)

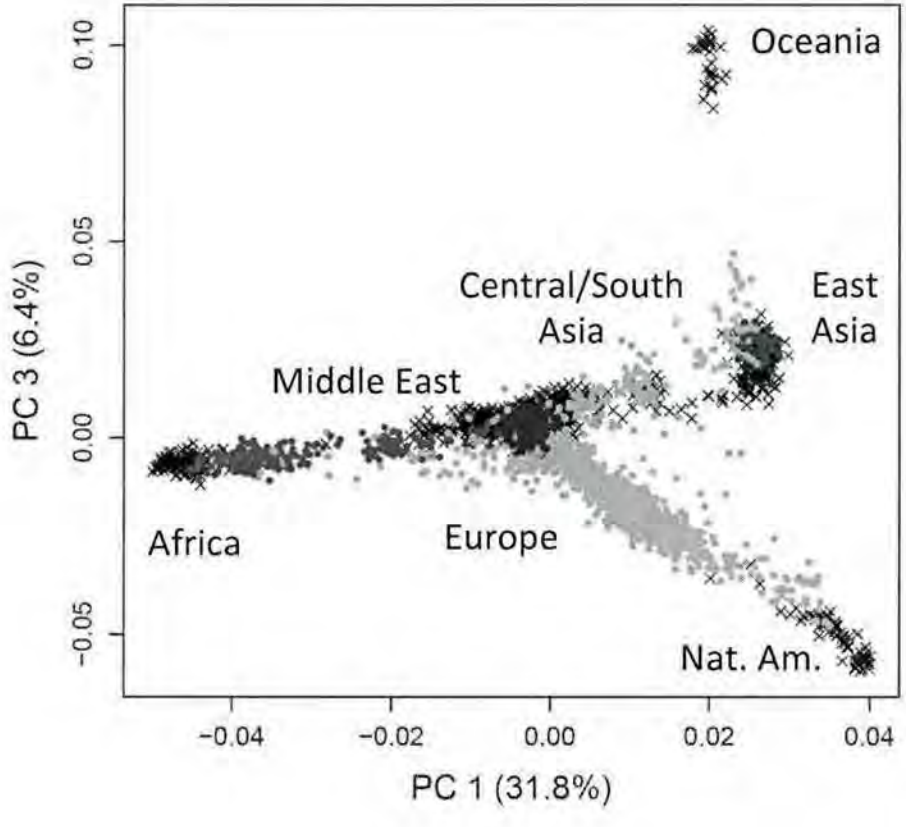
MRS: Genetic Ancestry

MRS subjects (N=3,669)
- African American n=218
- Hispanic/Nat.Am. N=671
- East Asians n=52
- Europeans n=2,287
- Others n=493

Reference Subjects
- × HGDP subjects

~62% European American





- Bayesian based cluster methods (STRUCTURE) to generate ancestry estimates based on HGDP reference populations and AIMs
- Determination of main ancestral groups (<5% admixture)
- Visual inspection: PCA with reference populations and color coding for main ancestral groups

GWAS study design

Psychoneuroendocrinology (2015)
journal homepage: www.elsevier.com/locate/psyneuen

Diagnosis: Partial and full PTSD (highest CAPS scores during study)

Genotypes: Imputed (SNP dosage); MAF>1%

Statistical analysis: logistic regressions for each ancestry group

Covariates: GWAS platform, cohort, 5PCs

Meta-analysis: fixed-effects inverse-variance weighted (METAL)

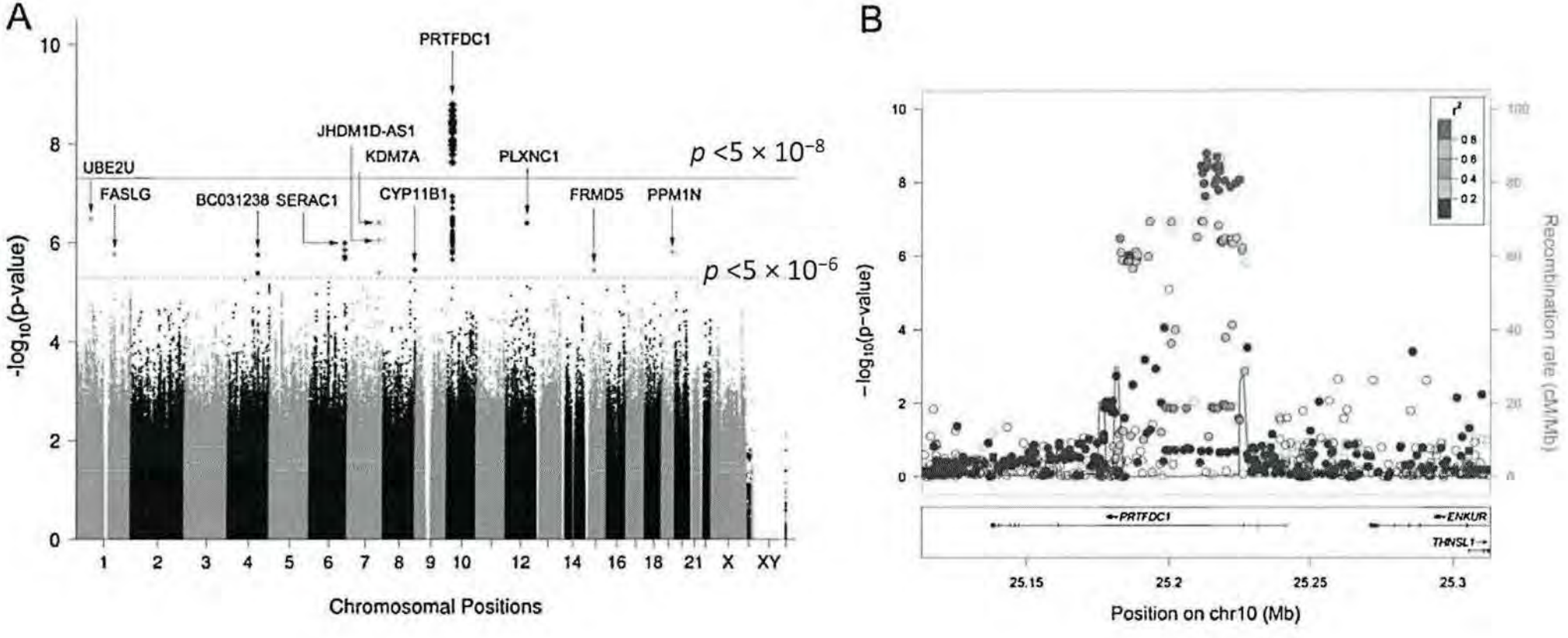
Genomic predictors of combat stress vulnerability and resilience in U.S. Marines: A genome-wide association study across multiple ancestries implicates *PRTFDC1* as a potential PTSD gene

Table 1 Descriptive statistics for the Marine Resiliency GWAS cohorts (MRS) studied based on PTSD case versus control status.

	All	MRS-I	MRS-II	PTSD	Controls	*p*-Value*
Number of Subjects	3494	2376	1118	940	2554	
Age, mean (±SD)	23.1 (3.4)	23.3 (3.5)	22.6 (3.0)	23.0 (3.0)	23.2 (3.5)	0.98
Range	18–48	18–48	18–43	18–38	18–48	
Self reported race						
White	85.5%	84.6%	87.5%	84.1%	86.1%	0.23
African American	4.4%	4.5%	4.1%	4.4%	4.4%	
Other	10.0%	10.8%	8.4%	11.5%	9.5%	
Self reported ethnicity						
Hispanic	24.5%	23.3%	26.2%	25.9%	23.6%	0.16
Non-Hispanic	75.5%	76.7%	73.8%	74.1%	76.4%	
CTQ, mean (±SD)	39.6 (13.5)	40.3 (13.8)	38.0 (12.3)	44.3 (12.8)	37.8 (12.3)	$<2.2 \times 10^{-16}$
Range	25.0–107.5	25.0–106.5	25.0–107.5	25.0–106.5	25.0–107.5	
LEC, mean (±SD)	6.9 (3.5)	6.7 (3.5)	5.8 (3.3)	8.2 (3.4)	5.7 (3.3)	$<2.2 \times 10^{-16}$
Range	0–16	0–16	0–16	0–16	0–16	
Prior deployment	78%	78%	78%	83%	76%	1.4×10^{-5}

* *p*-Values (PTSD versus Controls) based on Wilcoxon tests (chi-square tests for Race and Ethnicity). CTQ, childhood trauma questionnaire; LEC: life events checklist.

Genome-wide significant association for SNPs in the phosphoribosyl transferase domain containing 1 gene (*PRTFDC1*)



(A) Manhattan plot of genome-wide association results for PTSD from a meta-analysis of subjects from mixed ancestries

(B) Regional association plot. The color of each circle is based on R^2 with rs6482463 and recombination rates are based on European reference subjects from the 1K Genomes Project. The region of the associated LD block (40kb in intron 3) shows enrichment in H3K27Ac and H3K4Me3 histone marks, indicative of high transcriptional activity.

Similar effect of SNPs in *PRTFDC1* on PTSD across MRS ancestry groups and an independent military replication sample (NCPTS)

Table 2 Meta-analyses of *PRTFDC1* associations with PTSD for (A) the most significant imputed SNP rs6482463 in four Marine Resiliency Study (MRS) ancestry groups, and (B) for the genotyped SNP rs1033962 in MRS and an independent replication sample from the National Center for PTSD/Boston (NCPTS).

Study	Ancestry	A1	A2	MAF	N subjects	OR	SE	P	Q
(A) Association analysis for rs6482463									
MRS	EA	A	G	0.22	2179	1.41	0.08	2.98×10^{-05}	
	AA	A	G	0.46	205	1.49	0.26	0.118	
	HNA	A	G	0.31	640	1.58	0.14	1.25×10^{-03}	
	OTH	A	G	0.31	470	1.55	0.18	0.012	
Meta		A	G	–	3494	1.47	0.06	2.04×10^{-09}	0.90
(B) Association analysis for rs1033962									
MRS	EA	A	G	0.22	2179	1.40	0.08	4.48×10^{-05}	
	AA	A	G	0.47	205	1.45	0.25	0.148	
	HNA	A	G	0.31	640	1.57	0.14	1.37×10^{-03}	
	OTH	A	G	0.31	470	1.52	0.17	0.016	
Meta	All	A	G	–	3494	1.45	0.06	4.93×10^{-09}	0.90
NCPTS	EA	A	G	0.21	491	1.28	0.17	0.144	
Meta	All	A	G	–	3985	1.43	0.06	2.06×10^{-09}	0.91

MAF, minor allele frequency for A1 allele; OR, odds ratio; SE, standard error of the mean; *Q*, *p*-value for Cochran's *Q* statistic; meta, inverse-variance weighted meta-analysis; EA, European American; AA, African American; HNA, Hispanic and Native American descent; OTH, other.

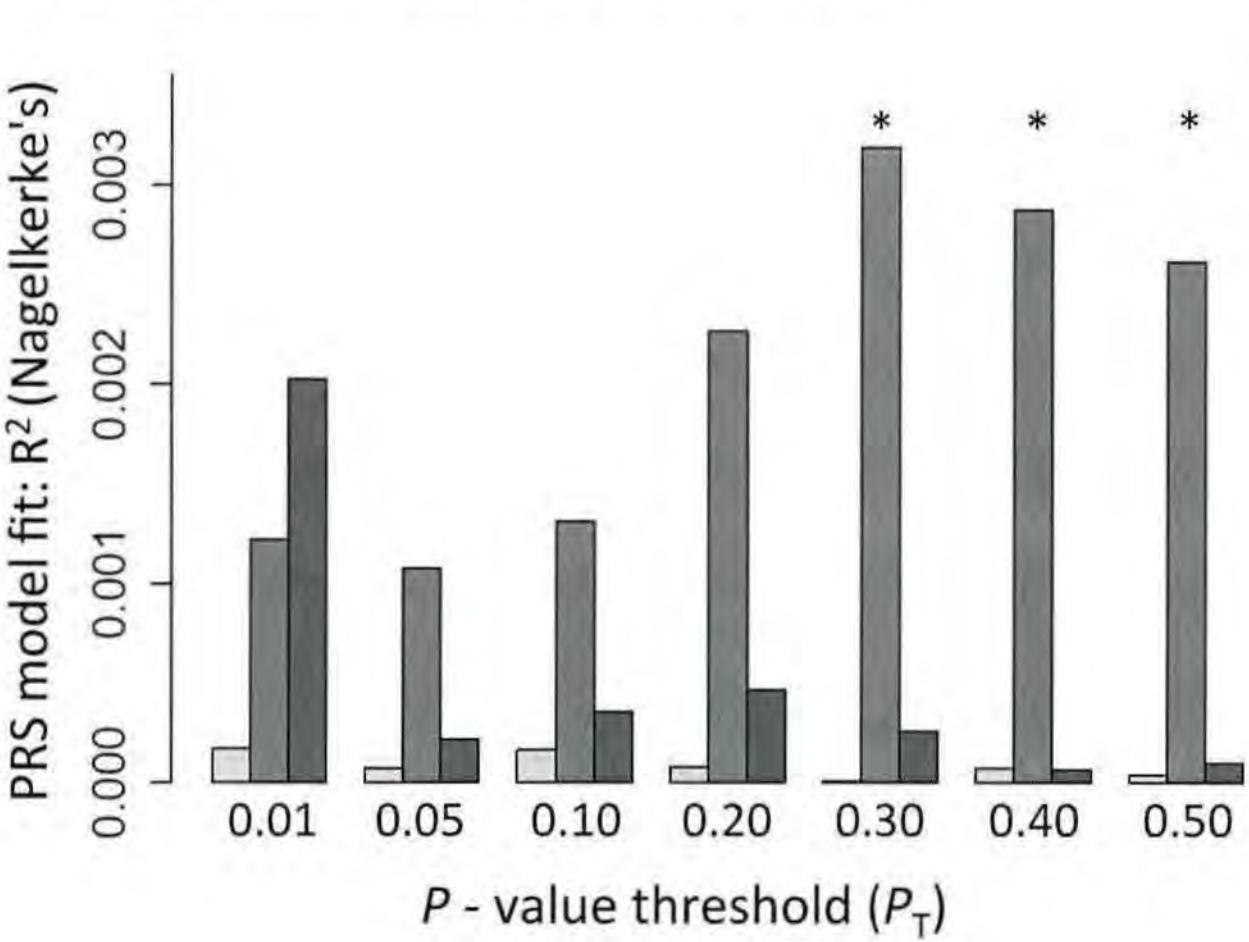
PRTFDC1 function

- encodes the phosphoribosyltransferase domain-containing protein 1
- small protein with highest expression in brain
- GO annotations: magnesium ion binding and protein homodimerization activity
- Paralog of HPRT1, associated with Lesch-Nyhan syndrome (uric acid)
- not yet been implicated in GWAS of PTSD or other psychiatric disorders
- its potential role in the etiology of PTSD remains to be determined

Polygenic Risk Score (PRS) analyses:

Can PRS from other psychiatric disorders predict PTSD status in MRS?

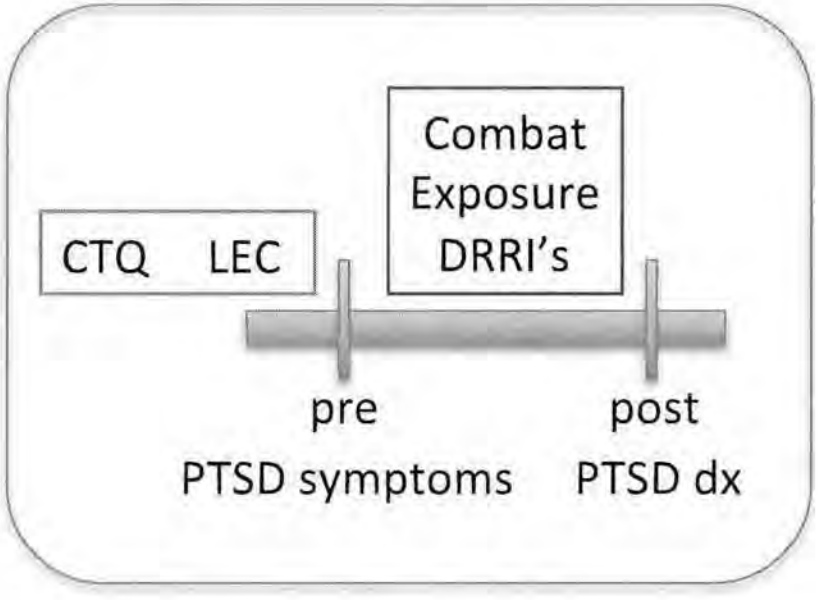
- PRS data from the PGC for MDD, BPD, and SCZ
- A risk score for each MRS participant was computed by the number of risk alleles weighted by the log of the odds ratios
- Logistic regression to predict PTSD status in MRS



Best fit for BPD:
$P_T = 0.36$
$p_{nominal} = 0.017$
(PRSice)

MRS analyses:
EA only: N = 2,287
Study max CAPS
Partial and full PTSD dx

GWAS Study design: PTSD symptom changes



Goal: identification of SNPs associated with larger changes in trauma-related symptoms than predicted by the severity of combat trauma exposure

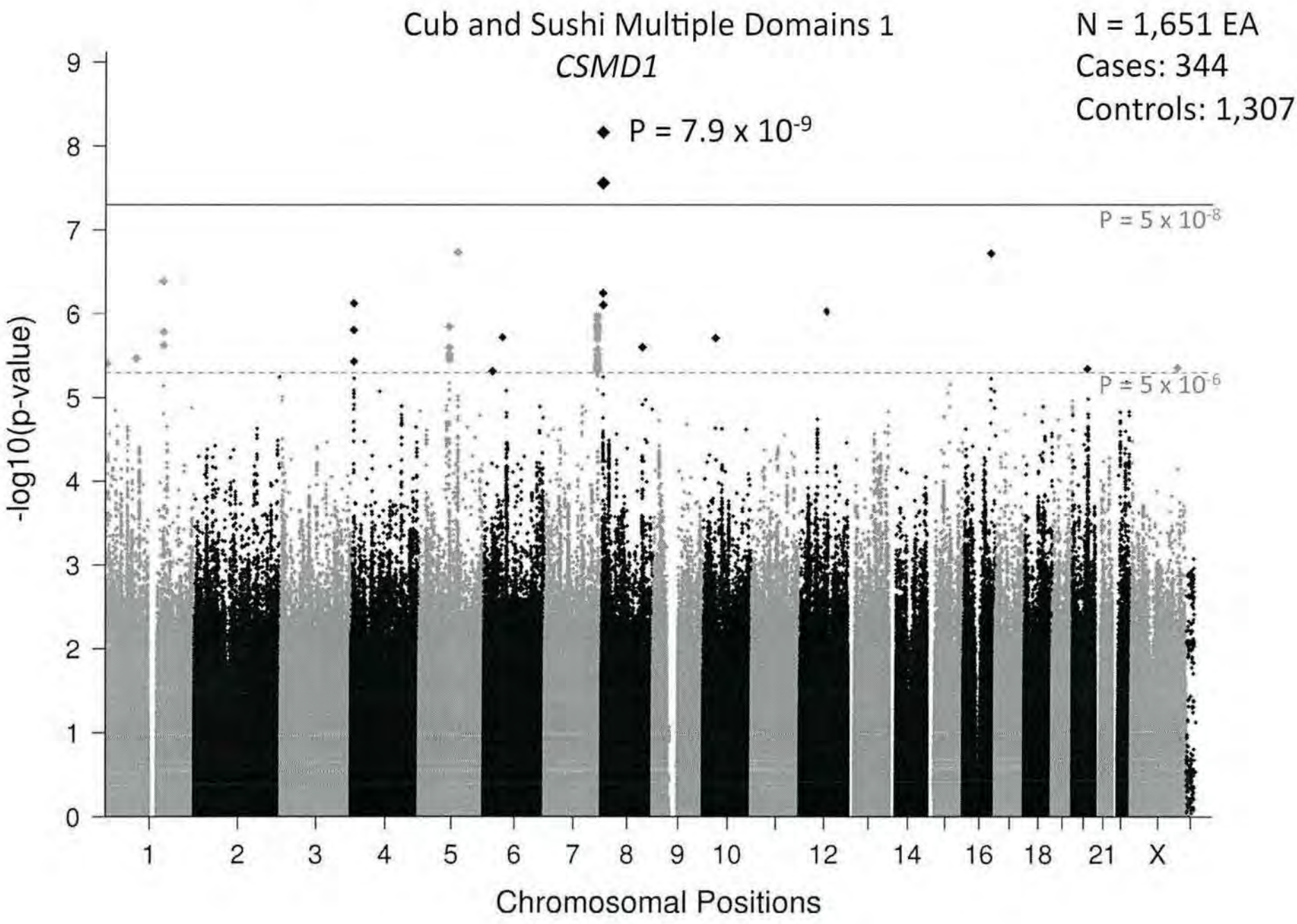
Post-deployment PTSD symptoms as predicted by pre-deployment PTSD and trauma measures:

Variable	% VE	P-value	Cum. % VE	P-cum.
CAPS V0	2.80%	< 2.2e-16	2.80%	< 2.2e-16
DRRI's	2.70%	< 2.2e-16	5.61%	< 2.2e-16
CTQ	1.19%	< 2.2e-16	5.92%	8.92E-10
LEC	0.74%	< 2.2e-16	6.01%	0.0023

DRRI's: composite score of combat exposure measures
CTQ: Childhood Trauma Questionnaire
LEC: Life Events Checklist at pre-deployment (V0)
%VE: % variability explained; cum.: cumulative

Predictors:
- CAPS V0
- DRRI's
- MRS study
- 3 PC's

Post-deployment PTSD case-control GWAS



Secondary GWAS models

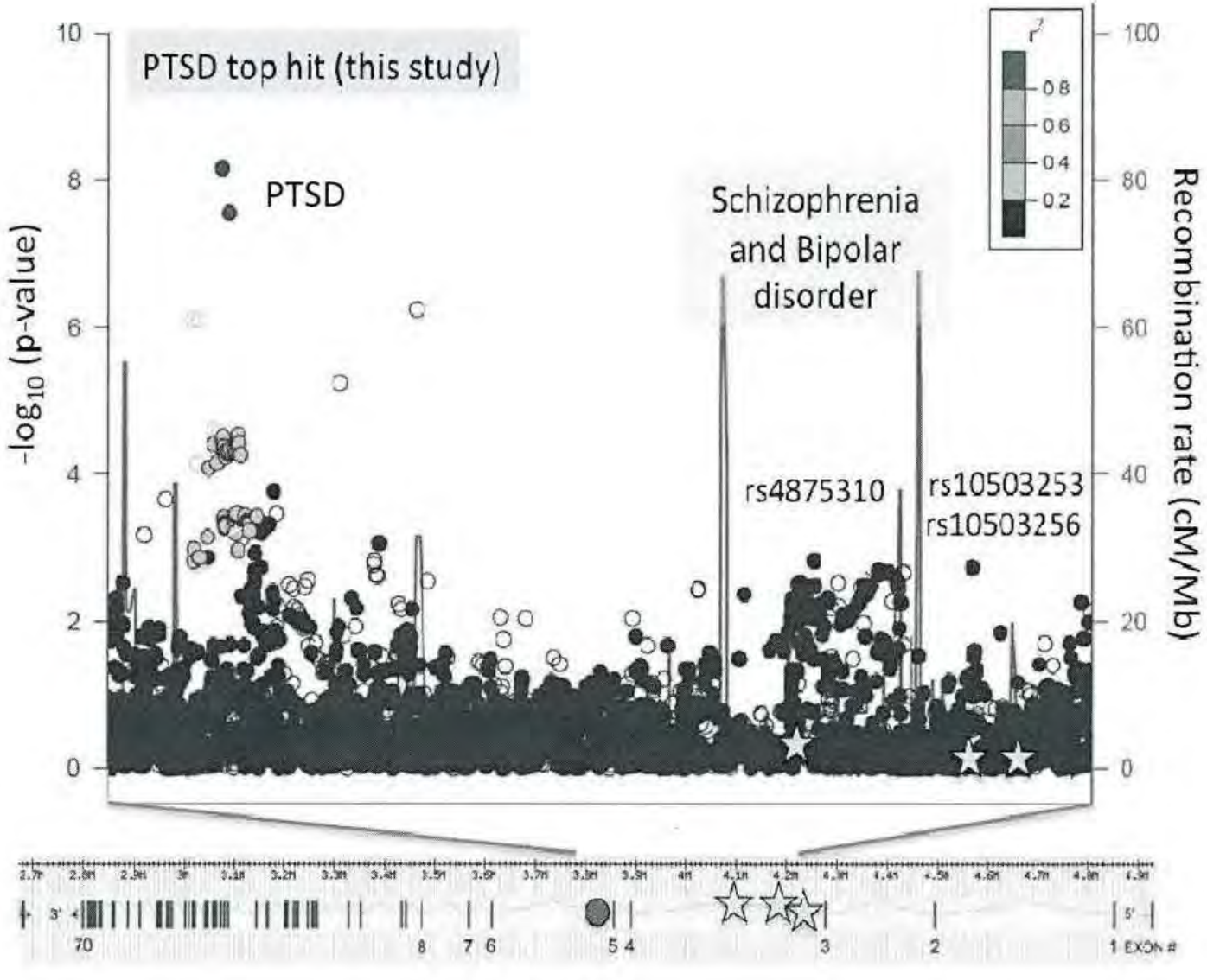
Variable	OR	SE	p	N	AIC	Model
SNP	2.333	0.146	**6.84E-09**	1651	1357.80	base
DRRI's	3.231	0.100	5.40E-32			
caps-qt V0	1.048	0.004	9.42E-26			
SNP	2.372	0.147	**4.66E-09**	1631	**1354.93**	adding CTQ
DRRI's	3.181	0.101	3.54E-30			
caps-qt V0	1.046	0.005	1.55E-22			
CTQ	1.376	0.141	0.024			
SNP	2.398	0.148	**3.27E-09**	1629	1354.95	adding CTQ & LEC
DRRI's	3.177	0.102	6.26E-30			
caps-qt V0	1.044	0.005	1.54E-19			
CTQ	1.326	0.143	0.048			
LEC	1.033	0.023	0.16			

- Adding more trauma variables increases gene effect on PTSD outcome
- Dominant genetic model is more significant than an additive one

CSMD1 function

- gene codes for a multiple domain complement-regulatory protein
- highly expressed in the central nervous system
- In rats:
  - CSMD1 protein blocked classical complement pathway activation
  - primary sites of synthesis are developing CNS and epithelial tissues, suggesting that CSMD1 may be a regulator of complement activation and inflammation in the developing CNS, and may also play a role in growth cone function (Kraus et al. 2006)
- *Csmd1* knockout mice showed behaviors reminiscent of blunted emotional responses, anxiety and depression, suggesting an influence of CSMD1 on psychopathology and endophenotypes of the negative symptom spectra in schizophrenia (Steen et al. 2013)

Role of *CSMD1* in Neuropsychiatric disorders



Gene size: 2MB

MRS Genomic Projects

- DNA and RNA isolated from peripheral blood leukocytes
- 60 (future) PTSD cases and 60 trauma-exposed controls
- Timepoints: at pre-deployment, and 3- and 6-months post-deployment
- Epigenome: Genome-wide methylation (Illumina 450K)
- Transcriptome: Genome-wide gene expression (RNAseq)
- Transcriptome 2: RNA array data on additional MRS subjects

RAPID PUBLICATION

AMERICAN JOURNAL OF
medical genetics
Neuropsychiatric Genetics

Blood-Based Gene-Expression Predictors of PTSD Risk and Resilience Among Deployed Marines: A Pilot Study

Stephen J. Glatt,[1*] Daniel S. Tylee,[1] Sharon D. Chandler,[2] Joel Pazol,[2] Caroline M. Nievergelt,[3,4,5] Christopher H. Woelk,[4,6] Dewleen G. Baker,[3,4,5] James B. Lohr,[3,4,5] William S. Kremen,[2,3,5] Brett T. Litz,[7] Ming T. Tsuang,[2,3,4,5,8,9,10] and Marine Resiliency Study Investigators

Molecular Psychiatry (2015), 1–8

www.nature.com/mp

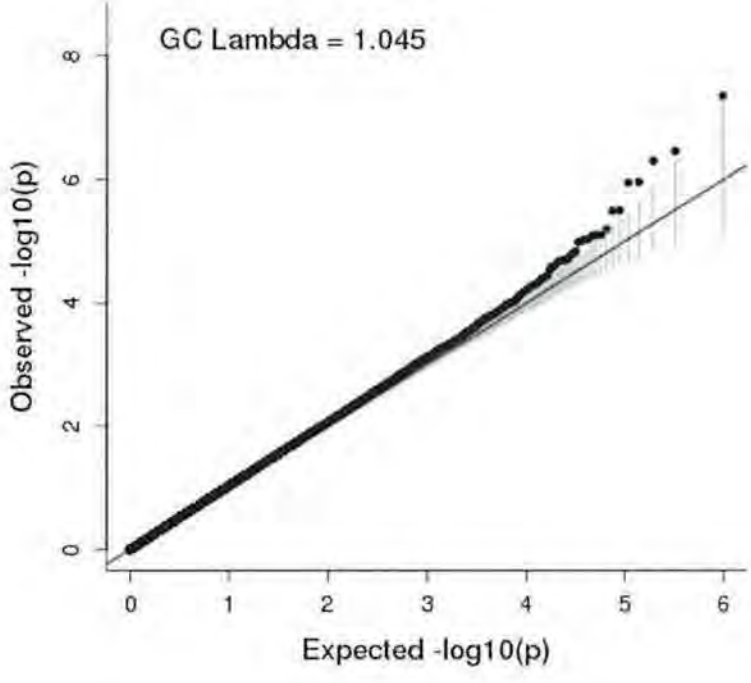
ORIGINAL ARTICLE

Gene networks specific for innate immunity define post-traumatic stress disorder

MS Breen[1], AX Maihofer[2], SJ Glatt[3], DS Tylee[3], SD Chandler[2], MT Tsuang[2,4,5,6,7], VB Risbrough[2,4], DG Baker[2,4], DT O'Connor[6,8], CM Nievergelt[2,4,9] and CH Woelk[1,9]

EWAS: Genome-wide methylation analyses

Example of ongoing epigenetic analyses:

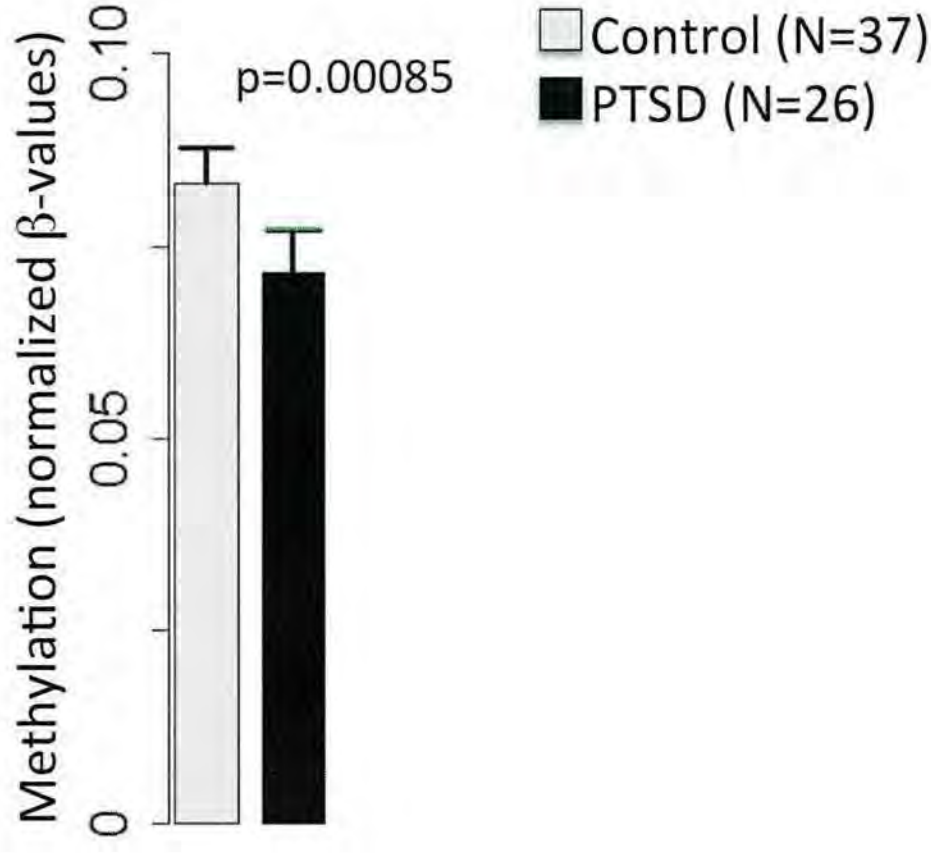


CpG	Beta	P	FDR	Gene
cg03118626	-0.403	4.43E-08	**0.02**	**KLK10**
cg22530232	-0.241	3.50E-07	0.08	PTPRN2
cg05818501	-0.757	4.98E-07	0.08	ADARB2
cg15577634	-0.340	1.10E-06	0.10	IFI30
cg00056257	-0.247	1.13E-06	0.10	GRM7

Replication analyses ongoing:
- PRISMO
- ArmySTARRS

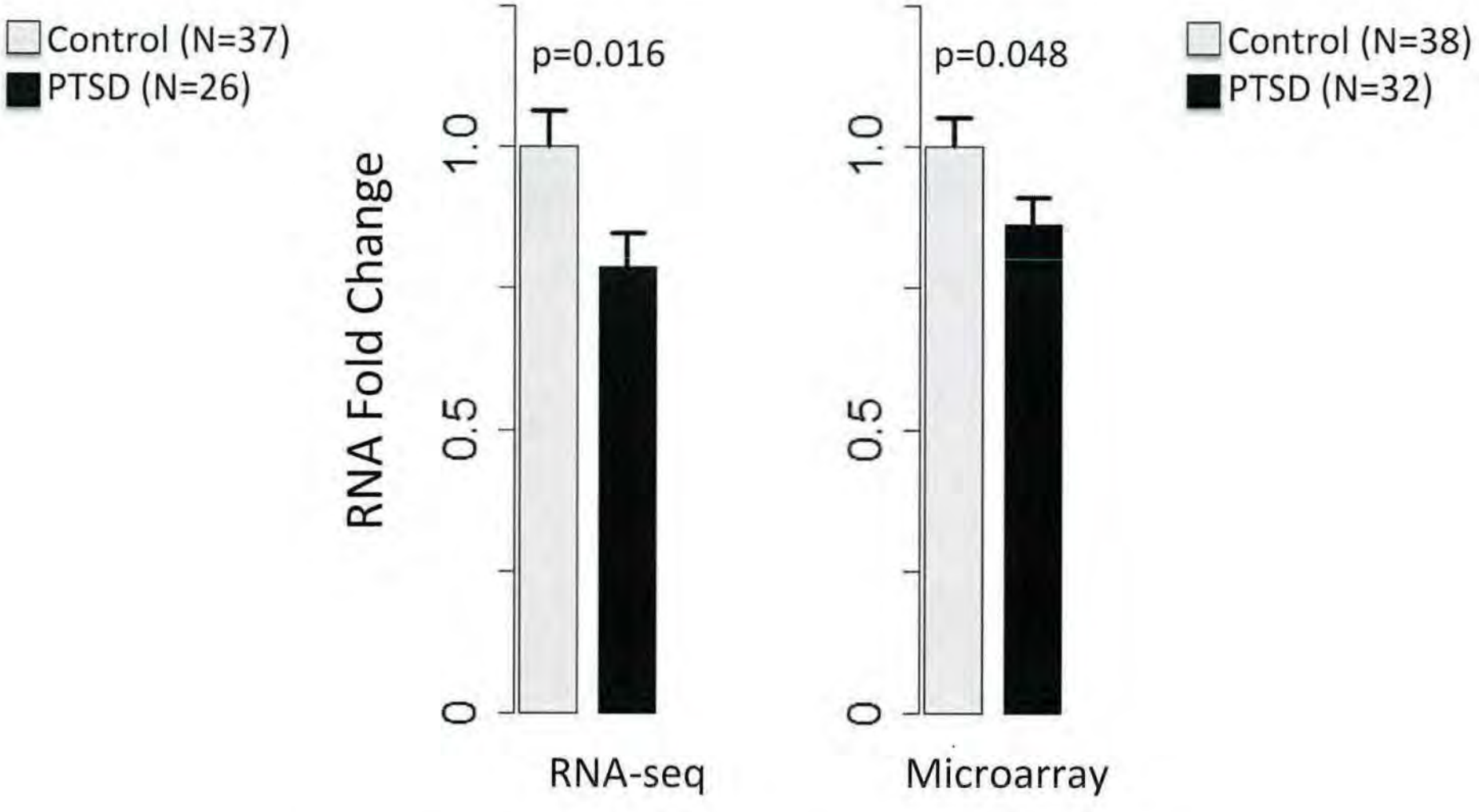
Subjects: 36 PTSD cases, 63 controls
At 6 months post-deployment
Conditioned on pre-deployment methylation and combat exposure

Mean (±SE) levels of methylation in CSMD1 probe is significantly lower in PTSD cases compared to controls three months after exposure to combat



Linear regression (β-regression) with predictors cases status, predeployment methylation, and 3 PC's

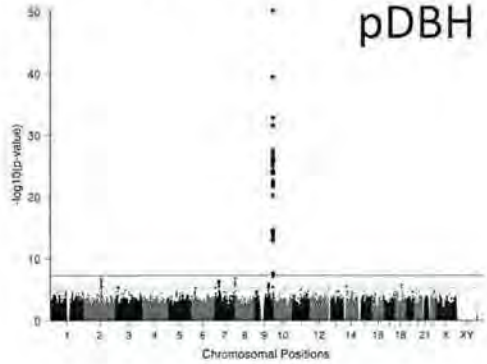
Significantly lower gene expression in CSMD1 in PTSD subjects compared to controls at post-deployment assessments



Data represent fold change ($2^{-\Delta VST}$) ± SE, relative to controls, following normalization and VST transformation

Mendelian Randomization in MRS

$p=7.2x10^{-51}$

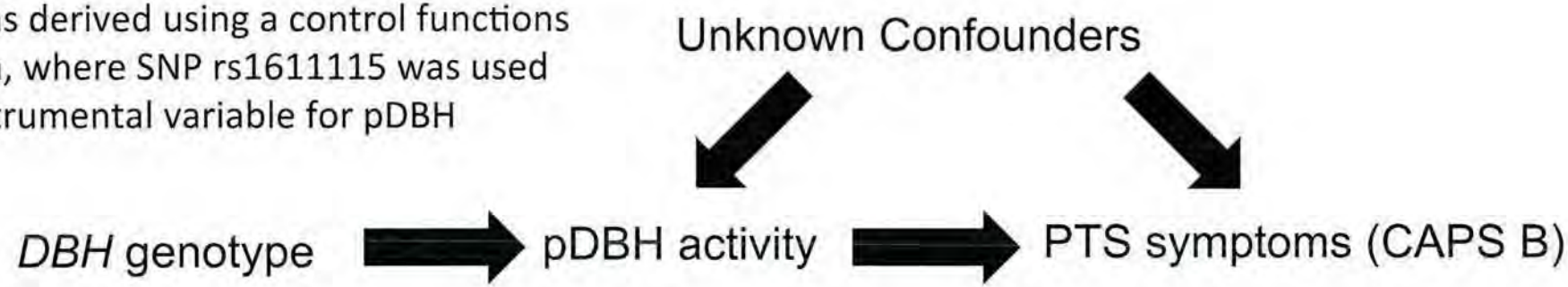


Dopamine beta-hydroxylase (DBH) catalyzes formation of norepinephrine and DBH activity was found to be associated with PTSD.

DBH as a causal predictor of PTS re-experiencing symptoms:

- pDBH levels and PTSD re-experiencing symptoms were found to be **positively associated** (p = 0.005)
- Top SNP rs1611115 was employed as a genetic instrument to test for a **causal effect** of pDBH on PTSD
- unconfounded estimate of the association of pDBH and re-experiencing symptoms **was significant** (beta=0.26, p = 0.002), indicating causality

MR estimates for the effect of pDBH on PTS symptoms derived using a control functions approach, where SNP rs1611115 was used as an instrumental variable for pDBH



Mustapic et al. (2014). The catecholamine biosynthetic enzyme dopamine β-hydroxylase(DBH): First genome-wide search positions trait-determining variants acting additively in the proximal promoter. Human Molecular Genetics.

Conclusion

- Genome-wide significant associations of *PRTFDC1* with PTSD
- Polygenic risk scores for bipolar disorder are associated with PTSD
- Genome-wide significant associations of *CSMD1* with PTSD symptom changes after exposure to combat
- Integration of methylome and transcriptome data to support GWAS findings
- MRS is a great resource (e.g. for Mendelian Randomization)

Collaborators and Support

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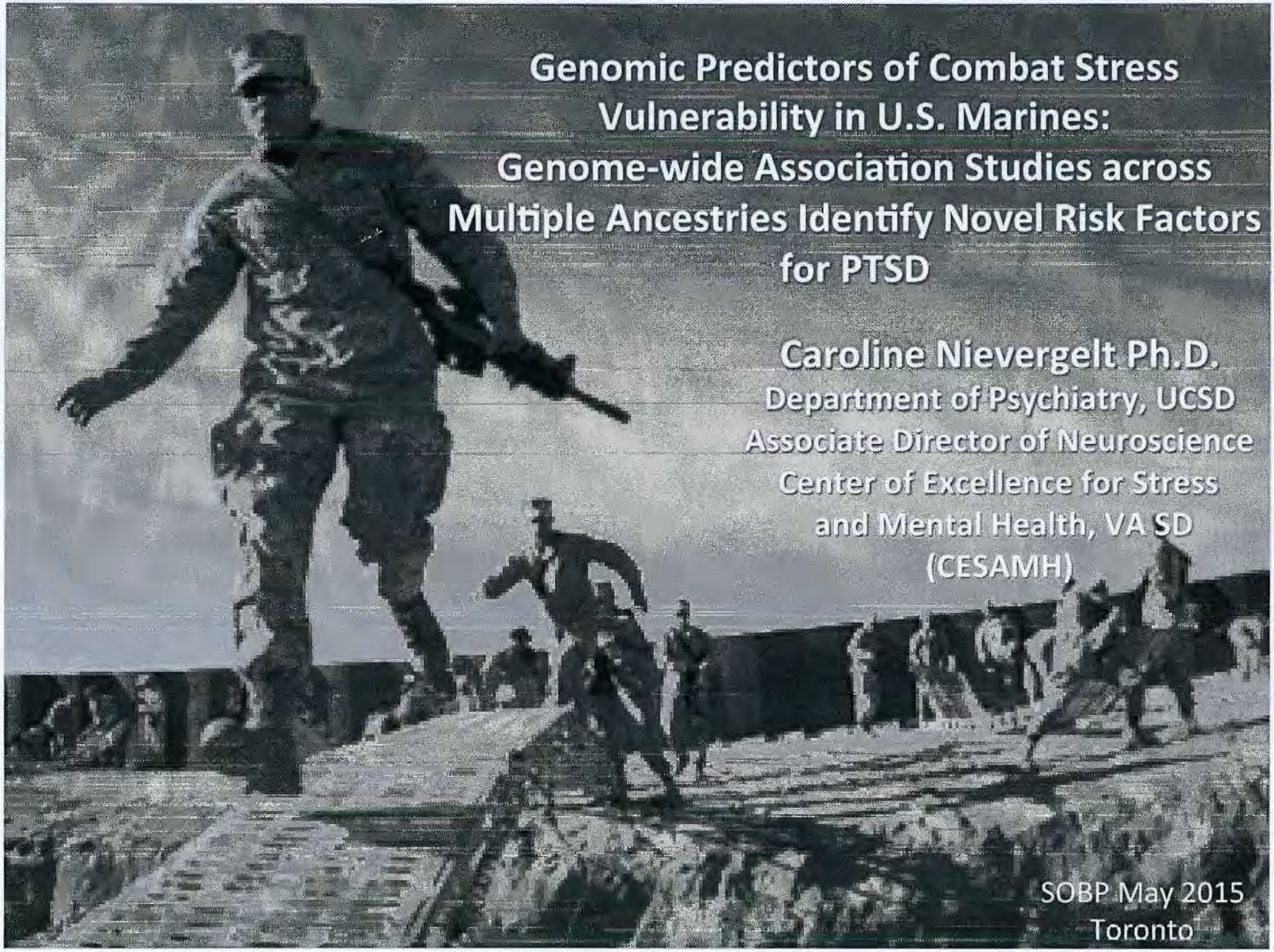
Baker Lab
Kate Yurgil
Andrew De La Rosa
Anjana Patel

Role of CSMD1 in Neuropsychiatric disorders

- GWAS **schizophrenia locus** (rs10503256) (SCZ Consortium 2011)
- Schizophrenia locus confirmed (rs10503253) (Bergen et al. 2012)
- GWAS showing supportive evidence for *CSMD1* in **bipolar disorder** (Xu et al. 2014)
- SNP rs10503256: associated with neurocognitive effects in humans, specifically with poorer performance on neuropsychological measures of general cognitive ability and memory function, **suggesting that CSMD1 may be involved in brain mechanisms related to memory and learning** (Donohoe et al. 2013)
- neural effects of rs10503253 were investigated in vivo in healthy participants in an MRI study, showing reduced cortical activations in the middle occipital gyrus and cuneus, suggesting that **CSMD1 may mediate brain function related to cognitive processes** (Rose et al. 2013)



Genomic Predictors of Combat Stress Vulnerability in U.S. Marines: Genome-wide Association Studies across Multiple Ancestries Identify Novel Risk Factors for PTSD

Caroline Nievergelt Ph.D.

Department of Psychiatry, UCSD

Associate Director of Neuroscience

Center of Excellence for Stress and Mental Health, VA SD (CESAMH)

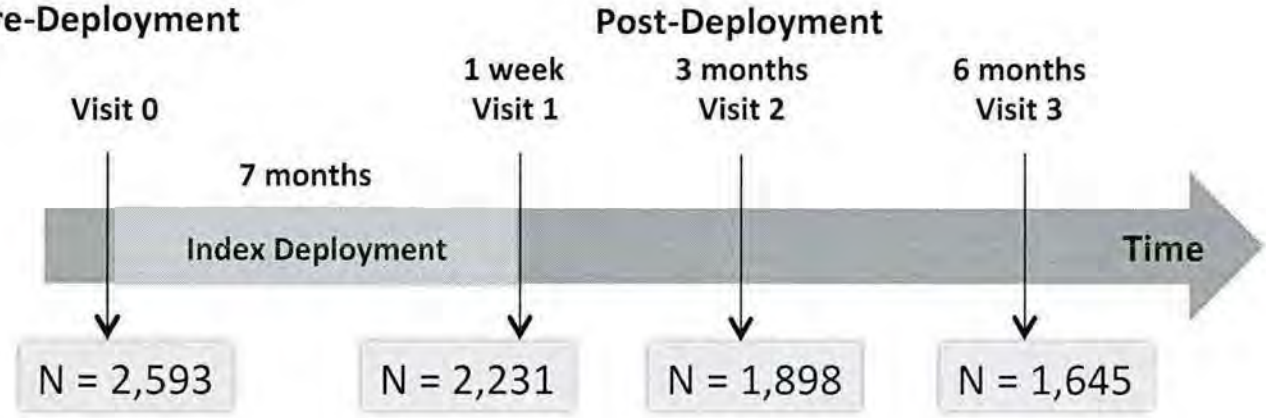
SOBP May 2015

Toronto

Marine Resiliency Study (MRS)

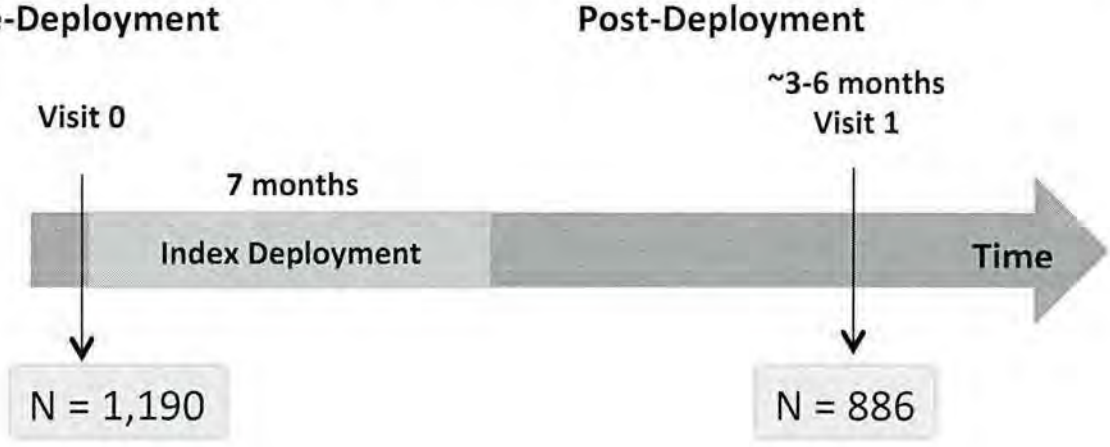
Goal: Predictors of Risk and Resilience for Posttraumatic Stress Disorder

MRS-I: 2008 – 2011 (PIs: Baker, Nash, Litz) Cohorts 1-4



- Prospective study
- Deployment to Iraq or Afghanistan
- Longitudinal follow-up

MRS-II: 2011 – 2013 (PIs: Baker, Risbrough, Geyer) Cohorts 11-12



Timeline and Enrollment

MRS Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews

Historical
- Self-report questionnaires

Neuropsychological
- Attention, Memory, Executive Function, Reasoning, Social Cognition

MRS Secure database

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

Biological assessments

Biomarkers
- NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Metabolomics (subset)

Telomere length (subset)

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- Startle threshold and habituation, fear conditioning and extinction, heart rate variability

Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Genomics

- **GWAS (complete data)**
- **Methylome (subset, pre-post)**
- **Transcriptome (subset, pre-post)**

PTSD diagnosis using CAPS

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(not for military cohorts)

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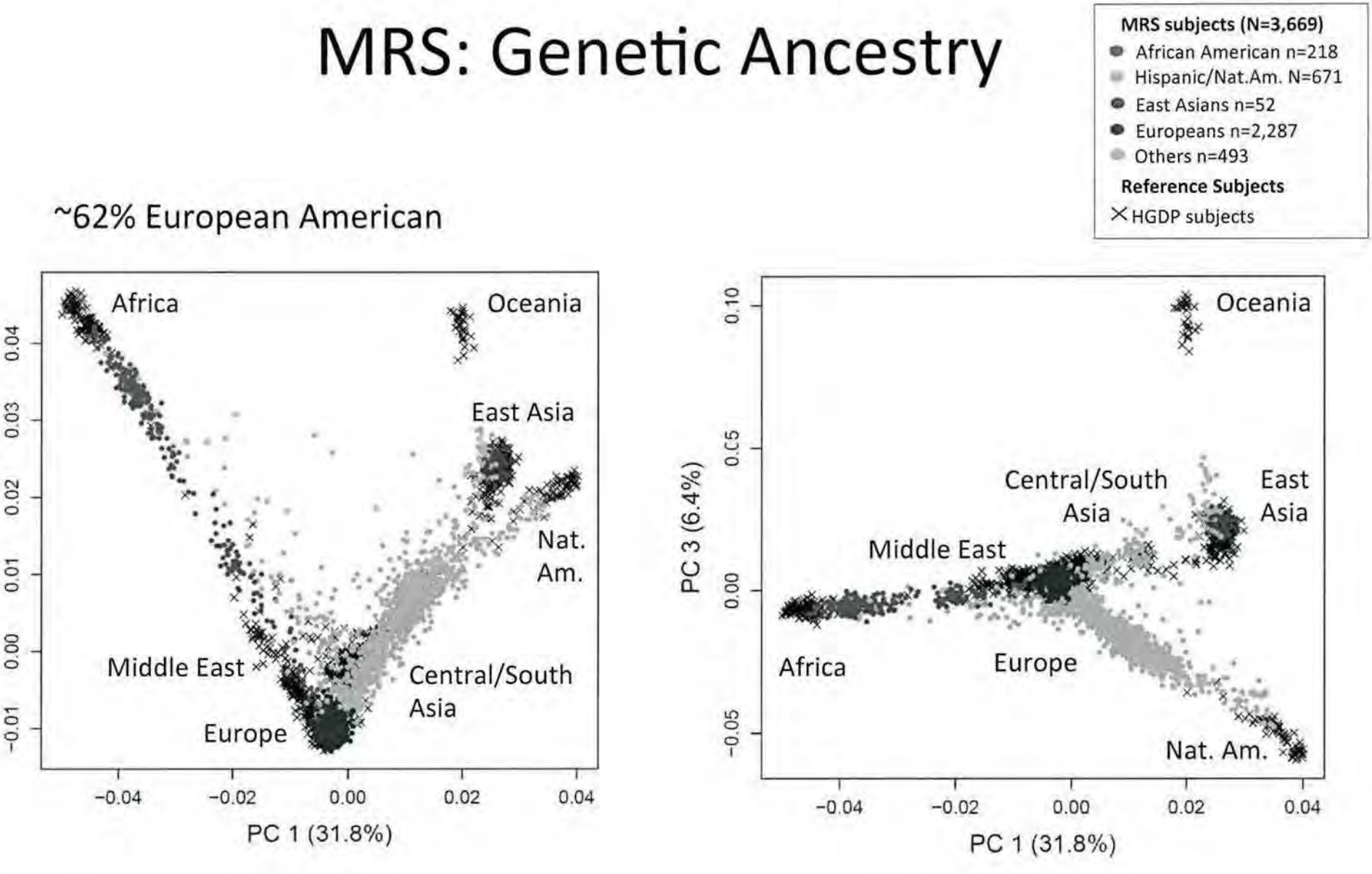
MRS: Genetic Ancestry



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Meta-analysis: fixed-effects inverse-variance weighted (METAL)

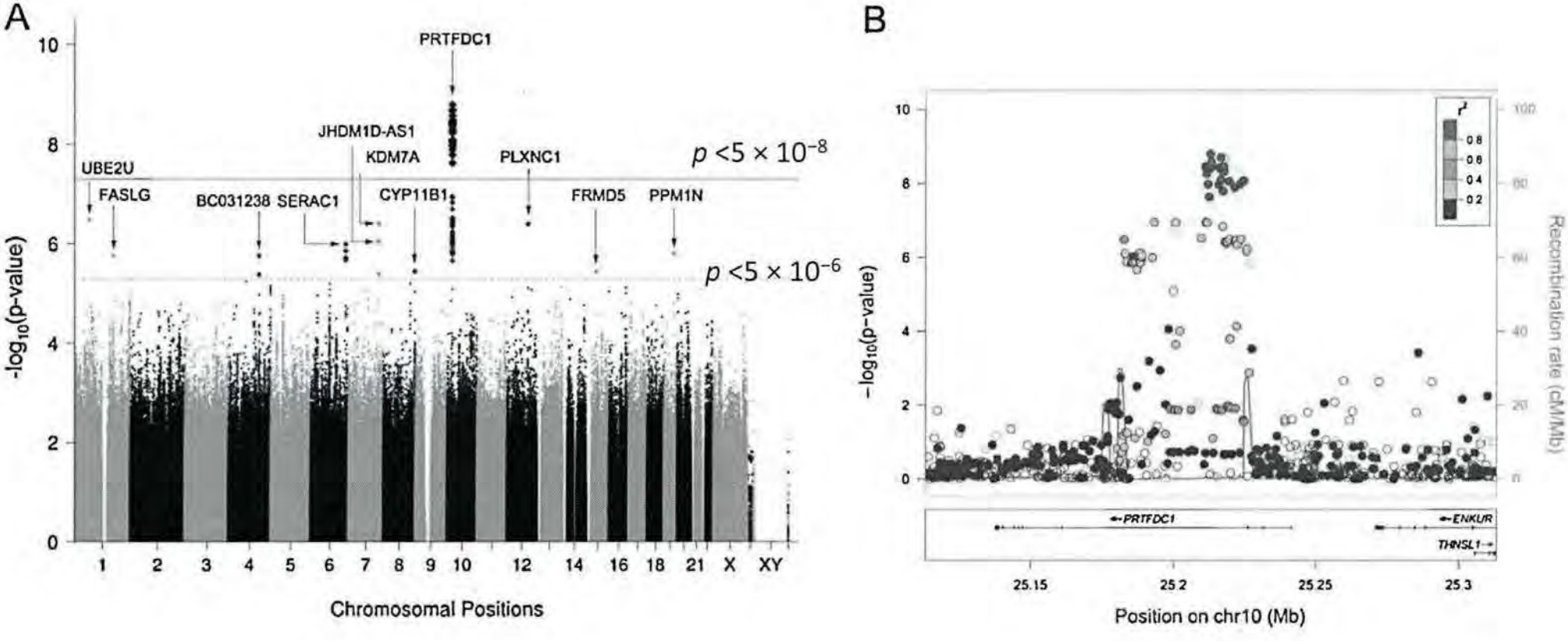
Genomic predictors of combat stress vulnerability and resilience in U.S. Marines: A genome-wide association study across multiple ancestries implicates *PRTFDC1* as a potential PTSD gene

Table 1 Descriptive statistics for the Marine Resiliency GWAS cohorts (MRS) studied based on PTSD case versus control status.

	All	MRS-I	MRS-II	PTSD	Controls	*p*-Value*
Number of Subjects	3494	2376	1118	940	2554	
Age, mean (±SD)	23.1 (3.4)	23.3 (3.5)	22.6 (3.0)	23.0 (3.0)	23.2 (3.5)	0.98
Range	18–48	18–48	18–43	18–38	18–48	
Self reported race						
White	85.5%	84.6%	87.5%	84.1%	86.1%	0.23
African American	4.4%	4.5%	4.1%	4.4%	4.4%	
Other	10.0%	10.8%	8.4%	11.5%	9.5%	
Self reported ethnicity						
Hispanic	24.5%	23.3%	26.2%	25.9%	23.6%	0.16
Non-Hispanic	75.5%	76.7%	73.8%	74.1%	76.4%	
CTQ, mean (±SD)	39.6 (13.5)	40.3 (13.8)	38.0 (12.3)	44.3 (12.8)	37.8 (12.3)	$<2.2 \times 10^{-16}$
Range	25.0–107.5	25.0–106.5	25.0–107.5	25.0–106.5	25.0–107.5	
LEC, mean (±SD)	6.9 (3.5)	6.7 (3.5)	5.8 (3.3)	8.2 (3.4)	5.7 (3.3)	$<2.2 \times 10^{-16}$
Range	0–16	0–16	0–16	0–16	0–16	
Prior deployment	78%	78%	78%	83%	76%	1.4×10^{-5}

* *p*-Values (PTSD versus Controls) based on Wilcoxon tests (chi-square tests for Race and Ethnicity). CTQ, childhood trauma questionnaire; LEC: life events checklist.

Genome-wide significant association for SNPs in the phosphoribosyl transferase domain containing 1 gene (*PRTFDC1*)



(A) Manhattan plot of genome-wide association results for PTSD from a meta-analysis of subjects from mixed ancestries

(B) Regional association plot. The color of each circle is based on R^2 with rs6482463 and recombination rates are based on European reference subjects from the 1K Genomes Project. The region of the associated LD block (40kb in intron 3) shows enrichment in H3K27Ac and H3K4Me3 histone marks, indicative of high transcriptional activity.

Similar effect of SNPs in *PRTFDC1* on PTSD across MRS ancestry groups and an independent military replication sample (NCPTS)

Table 2 Meta-analyses of *PRTFDC1* associations with PTSD for (A) the most significant imputed SNP rs6482463 in four Marine Resiliency Study (MRS) ancestry groups, and (B) for the genotyped SNP rs1033962 in MRS and an independent replication sample from the National Center for PTSD/Boston (NCPTS).

Study	Ancestry	A1	A2	MAF	N subjects	OR	SE	P	Q
(A) Association analysis for rs6482463									
MRS	EA	A	G	0.22	2179	1.41	0.08	2.98×10^{-05}	
	AA	A	G	0.46	205	1.49	0.26	0.118	
	HNA	A	G	0.31	640	1.58	0.14	1.25×10^{-03}	
	OTH	A	G	0.31	470	1.55	0.18	0.012	
Meta		A	G	–	3494	1.47	0.06	2.04×10^{-09}	0.90

MAF, minor allele frequency for A1 allele; OR, odds ratio; SE, standard error of the mean; *Q*, *p*-value for Cochran's *Q* statistic; meta, inverse-variance weighted meta-analysis; EA, European American; AA, African American; HNA, Hispanic and Native American descent; OTH, other.

Similar effect of SNPs in *PRTFDC1* on PTSD across MRS ancestry groups and an independent military replication sample (NCPTS)

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Meta		A	G	—	3494	1.47	0.06	2.04×10^{-09}	0.90
(B) Association analysis for rs1033962									
MRS	EA	A	G	0.22	2179	1.40	0.08	4.48×10^{-05}	
	AA	A	G	0.47	205	1.45	0.25	0.148	
	HNA	A	G	0.31	640	1.57	0.14	1.37×10^{-03}	
	OTH	A	G	0.31	470	1.52	0.17	0.016	
Meta	All	A	G	—	3494	1.45	0.06	4.93×10^{-09}	0.90
NCPTS	EA	A	G	0.21	491	1.28	0.17	0.144	
Meta	All	A	G	—	3985	1.43	0.06	2.06×10^{-09}	0.91

MAF, minor allele frequency for A1 allele; OR, odds ratio; SE, standard error of the mean; *Q*, *p*-value for Cochran's *Q* statistic; meta, inverse-variance weighted meta-analysis; EA, European American; AA, African American; HNA, Hispanic and Native American descent; OTH, other.

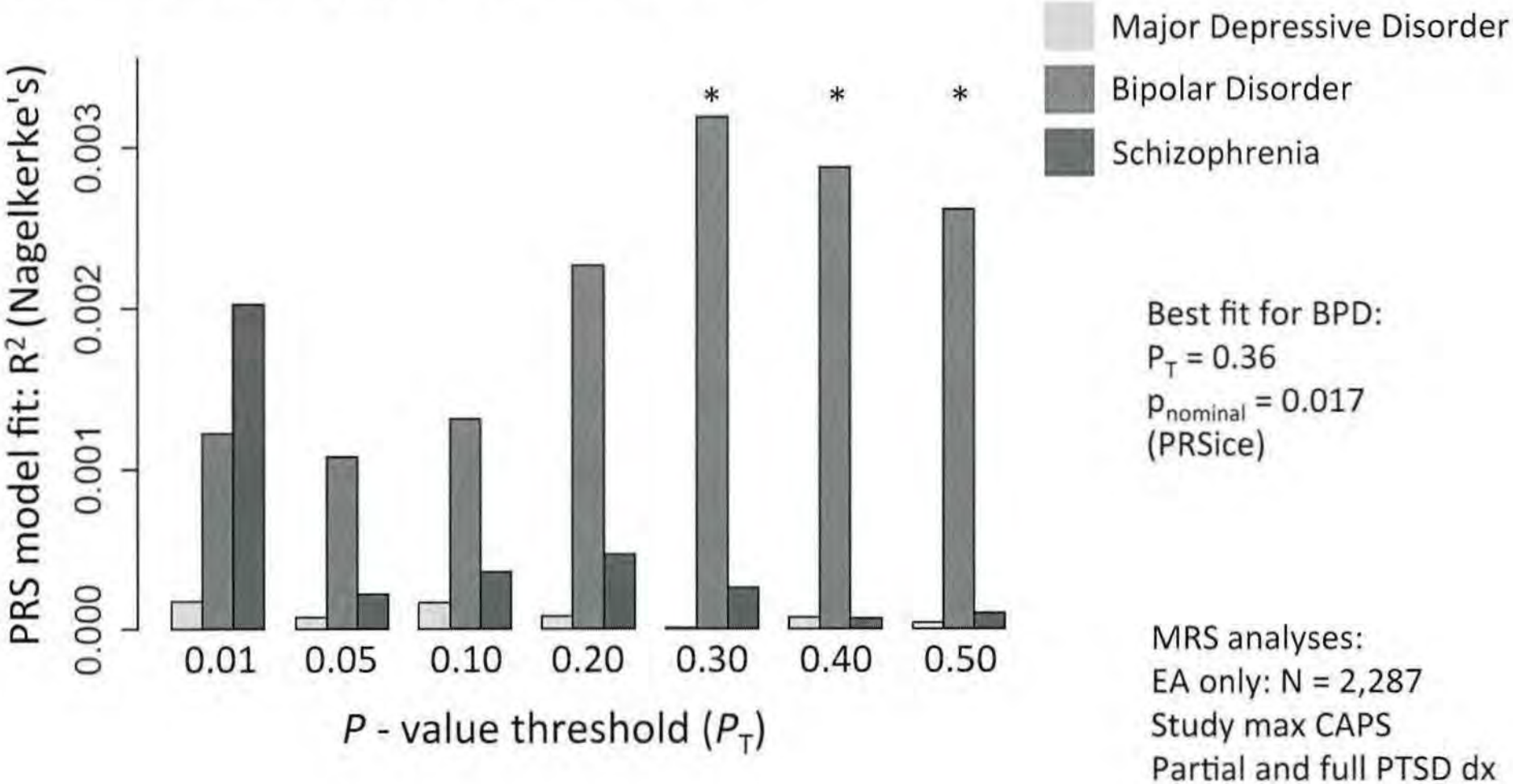
PRTFDC1 function

- encodes the phosphoribosyltransferase domain-containing protein 1
- small protein with highest expression in brain
- GO annotations: magnesium ion binding and protein homodimerization activity
- Paralog of HPRT1, associated with Lesch-Nyhan syndrome (uric acid)
- not yet been implicated in GWAS of PTSD or other psychiatric disorders
- its potential role in the etiology of PTSD remains to be determined

Polygenic Risk Score (PRS) analyses:

Can PRS from other psychiatric disorders predict PTSD status in MRS?

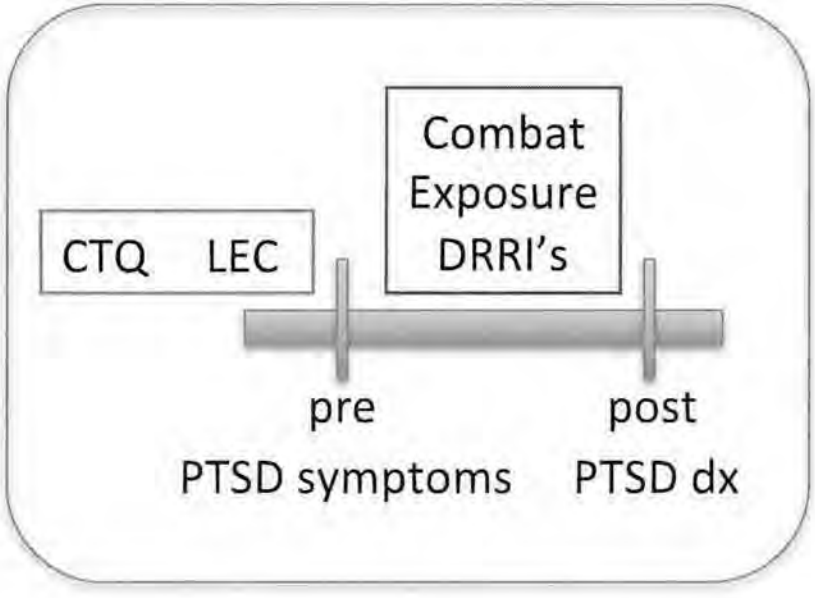
- PRS data from the PGC for MDD, BPD, and SCZ
- A risk score for each MRS participant was computed by the number of risk alleles weighted by the log of the odds ratios
- Logistic regression to predict PTSD status in MRS



Best fit for BPD:
$P_T = 0.36$
$p_{nominal} = 0.017$
(PRSice)

MRS analyses:
EA only: N = 2,287
Study max CAPS
Partial and full PTSD dx

GWAS Study design: PTSD symptom changes



Goal: identification of SNPs associated with larger changes in trauma-related symptoms than predicted by the severity of combat trauma exposure

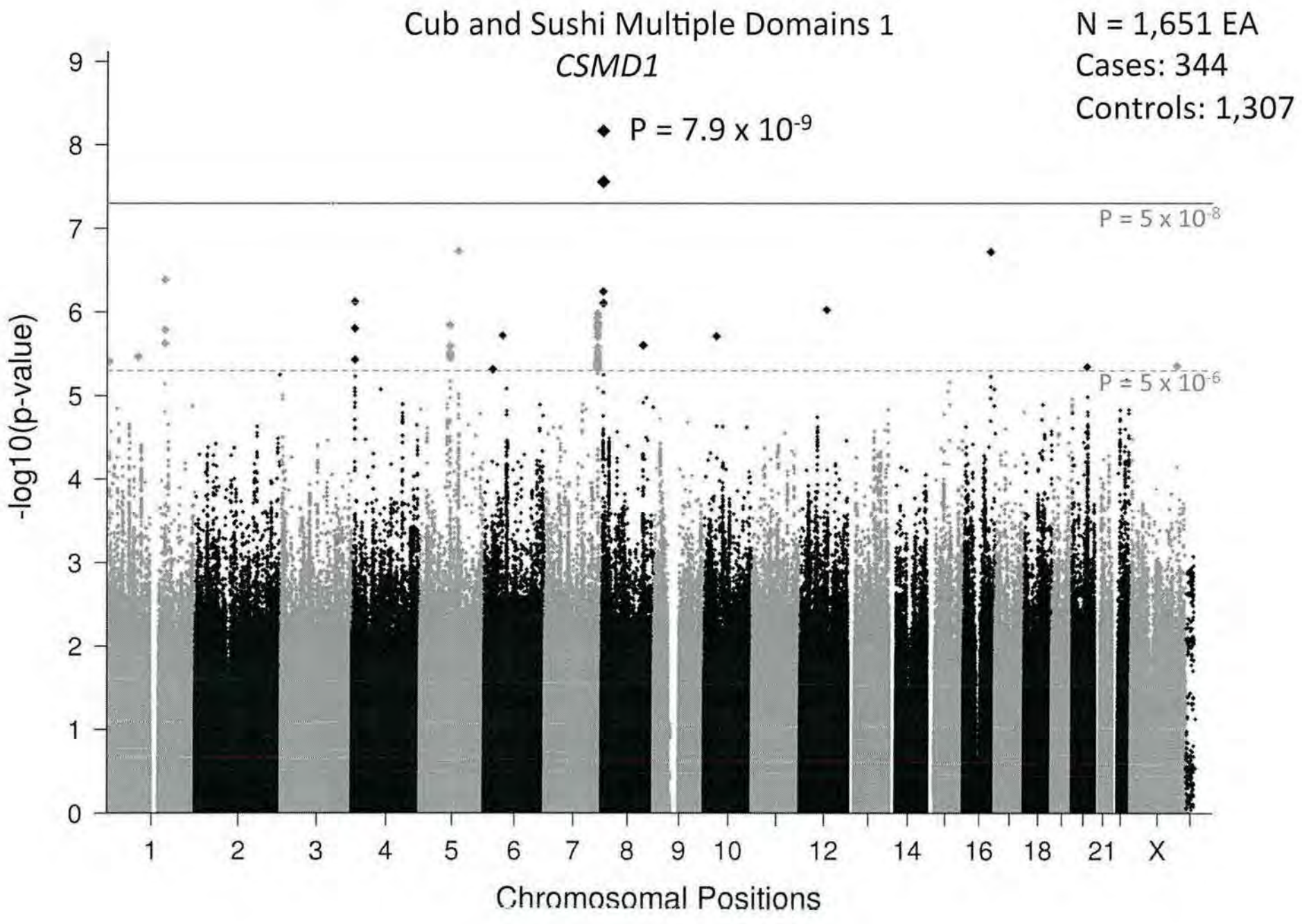
Post-deployment PTSD symptoms as predicted by pre-deployment PTSD and trauma measures:

Variable	% VE	P-value	Cum. % VE	P-cum.
CAPS V0	2.80%	< 2.2e-16	2.80%	< 2.2e-16
DRRI's	2.70%	< 2.2e-16	5.61%	< 2.2e-16
CTQ	1.19%	< 2.2e-16	5.92%	8.92E-10
LEC	0.74%	< 2.2e-16	6.01%	0.0023

DRRI's: composite score of combat exposure measures
CTQ: Childhood Trauma Questionnaire
LEC: Life Events Checklist at pre-deployment (V0)
%VE: % variability explained; cum.: cumulative

Predictors:
- CAPS V0
- DRRI's
- MRS study
- 3 PC's

Post-deployment PTSD case-control GWAS



Secondary GWAS models

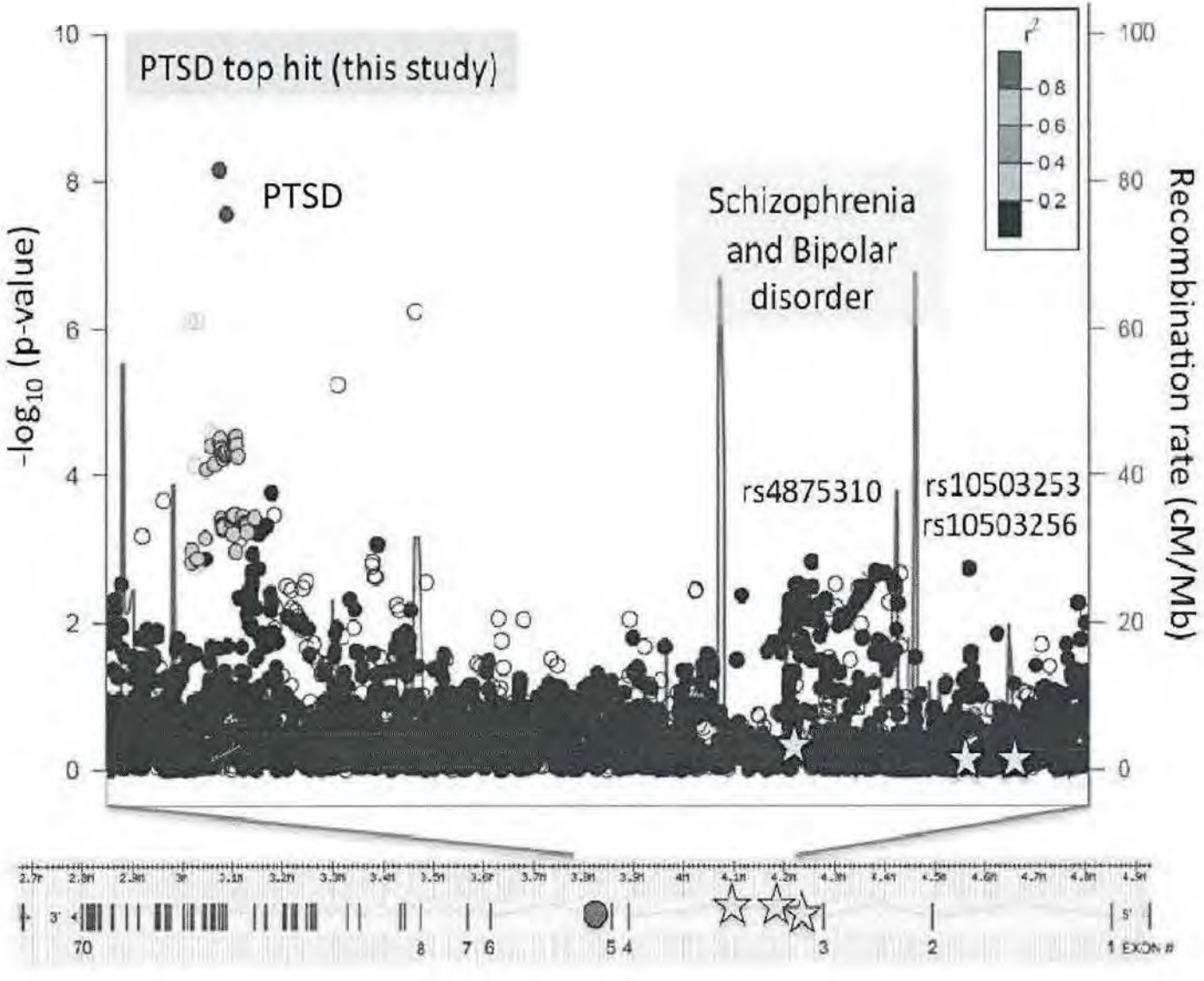
Variable	OR	SE	p	N	AIC	Model
SNP	2.333	0.146	**6.84E-09**	1651	1357.80	base
DRRI's	3.231	0.100	5.40E-32			
caps-qt V0	1.048	0.004	9.42E-26			
SNP	2.372	0.147	**4.66E-09**	1631	**1354.93**	adding CTQ
DRRI's	3.181	0.101	3.54E-30			
caps-qt V0	1.046	0.005	1.55E-22			
CTQ	1.376	0.141	0.024			
SNP	2.398	0.148	**3.27E-09**	1629	1354.95	adding CTQ & LEC
DRRI's	3.177	0.102	6.26E-30			
caps-qt V0	1.044	0.005	1.54E-19			
CTQ	1.326	0.143	0.048			
LEC	1.033	0.023	0.16			

- Adding more trauma variables increases gene effect on PTSD outcome
- Dominant genetic model is more significant than an additive one

CSMD1 function

- gene codes for a multiple domain complement-regulatory protein
- highly expressed in the central nervous system
- In rats:
  - CSMD1 protein blocked classical complement pathway activation
  - primary sites of synthesis are developing CNS and epithelial tissues, suggesting that CSMD1 may be a regulator of complement activation and inflammation in the developing CNS, and may also play a role in growth cone function (Kraus et al. 2006)
- *Csmd1* knockout mice showed behaviors reminiscent of blunted emotional responses, anxiety and depression, suggesting an influence of CSMD1 on psychopathology and endophenotypes of the negative symptom spectra in schizophrenia (Steen et al. 2013)

Role of *CSMD1* in Neuropsychiatric disorders



Gene size: 2MB

Role of CSMD1 in Neuropsychiatric disorders

- GWAS **schizophrenia locus** (rs10503256) (SCZ Consortium 2011)
- Schizophrenia locus confirmed (rs10503253) (Bergen et al. 2012)
- GWAS showing supportive evidence for *CSMD1* in **bipolar disorder** (Xu et al. 2014)
- SNP rs10503256: associated with neurocognitive effects in humans, specifically with poorer performance on neuropsychological measures of general cognitive ability and memory function, **suggesting that CSMD1 may be involved in brain mechanisms related to memory and learning** (Donohoe et al. 2013)
- neural effects of rs10503253 were investigated in vivo in healthy participants in an MRI study, showing reduced cortical activations in the middle occipital gyrus and cuneus, suggesting that **CSMD1 may mediate brain function related to cognitive processes** (Rose et al. 2013)

MRS Genomic Projects

- DNA and RNA isolated from peripheral blood leukocytes
- 60 (future) PTSD cases and 60 trauma-exposed controls
- Timepoints: at pre-deployment, and 3- and 6-months post-deployment
- Epigenome: Genome-wide methylation (Illumina 450K)
- Transcriptome: Genome-wide gene expression (RNAseq)
- Transcriptome 2: RNA array data on additional MRS subjects

RAPID PUBLICATION

AMERICAN JOURNAL OF medical genetics PART B
Neuropsychiatric Genetics

Blood-Based Gene-Expression Predictors of PTSD Risk and Resilience Among Deployed Marines: A Pilot Study

Stephen J. Glatt,[1*] Daniel S. Tylee,[1] Sharon D. Chandler,[2] Joel Pazol,[2] Caroline M. Nievergelt,[3,4,5] Christopher H. Woelk,[4,6] Dewleen G. Baker,[3,4,5] James B. Lohr,[3,4,5] William S. Kremen,[2,3,5] Brett T. Litz,[7] Ming T. Tsuang,[2,3,4,5,8,9,10] and Marine Resiliency Study Investigators

Molecular Psychiatry (2015), 1–8

www.nature.com/mp

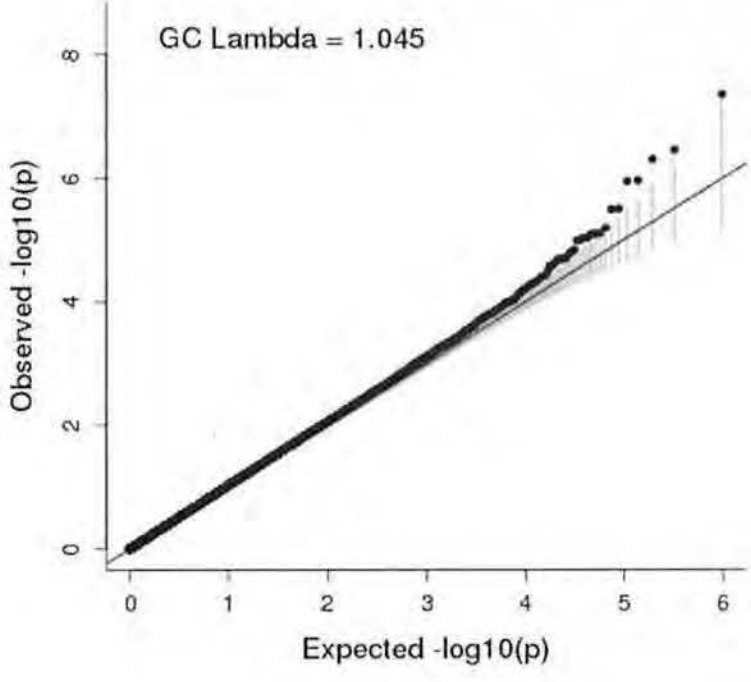
ORIGINAL ARTICLE

Gene networks specific for innate immunity define post-traumatic stress disorder

MS Breen[1], AX Maihofer[2], SJ Glatt[3], DS Tylee[3], SD Chandler[2], MT Tsuang[2,4,5,6,7], VB Risbrough[2,4], DG Baker[2,4], DT O'Connor[4,8], CM Nievergelt[2,4,9] and CH Woelk[1,9]

EWAS: Genome-wide methylation analyses

Example of ongoing epigenetic analyses:

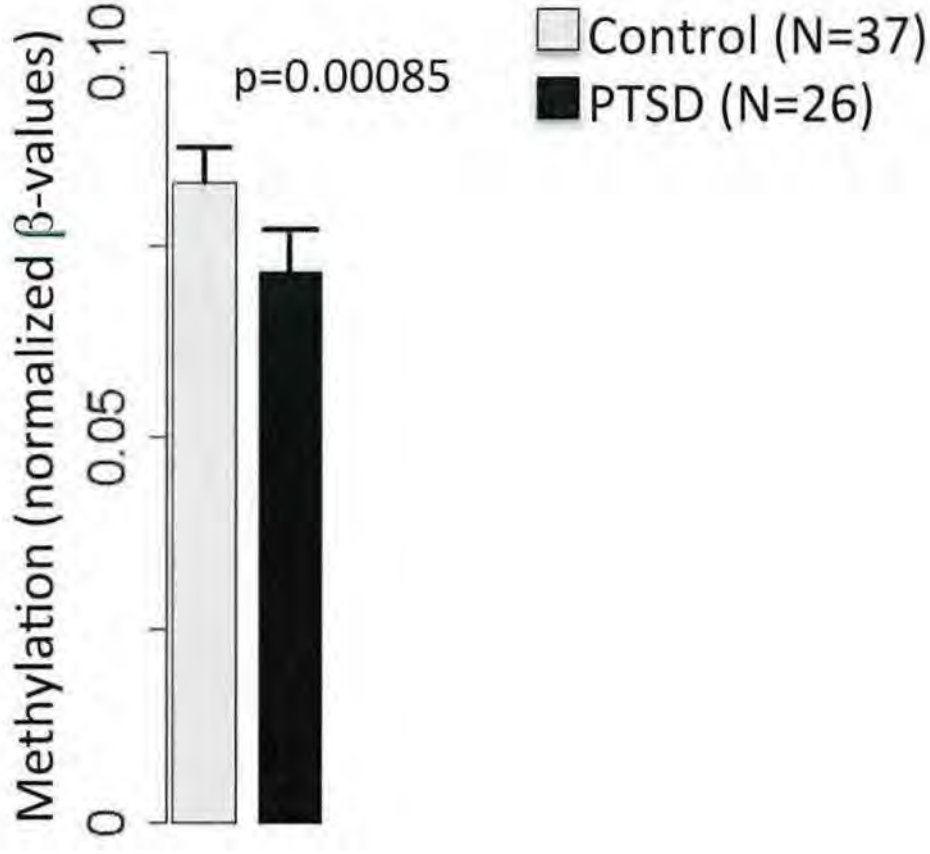


CpG	Beta	P	FDR	Gene
cg03118626	-0.403	4.43E-08	**0.02**	**KLK10**
cg22530232	-0.241	3.50E-07	0.08	PTPRN2
cg05818501	-0.757	4.98E-07	0.08	ADARB2
cg15577634	-0.340	1.10E-06	0.10	IFI30
cg00056257	-0.247	1.13E-06	0.10	GRM7

Replication analyses ongoing:
- PRISMO
- ArmySTARRS

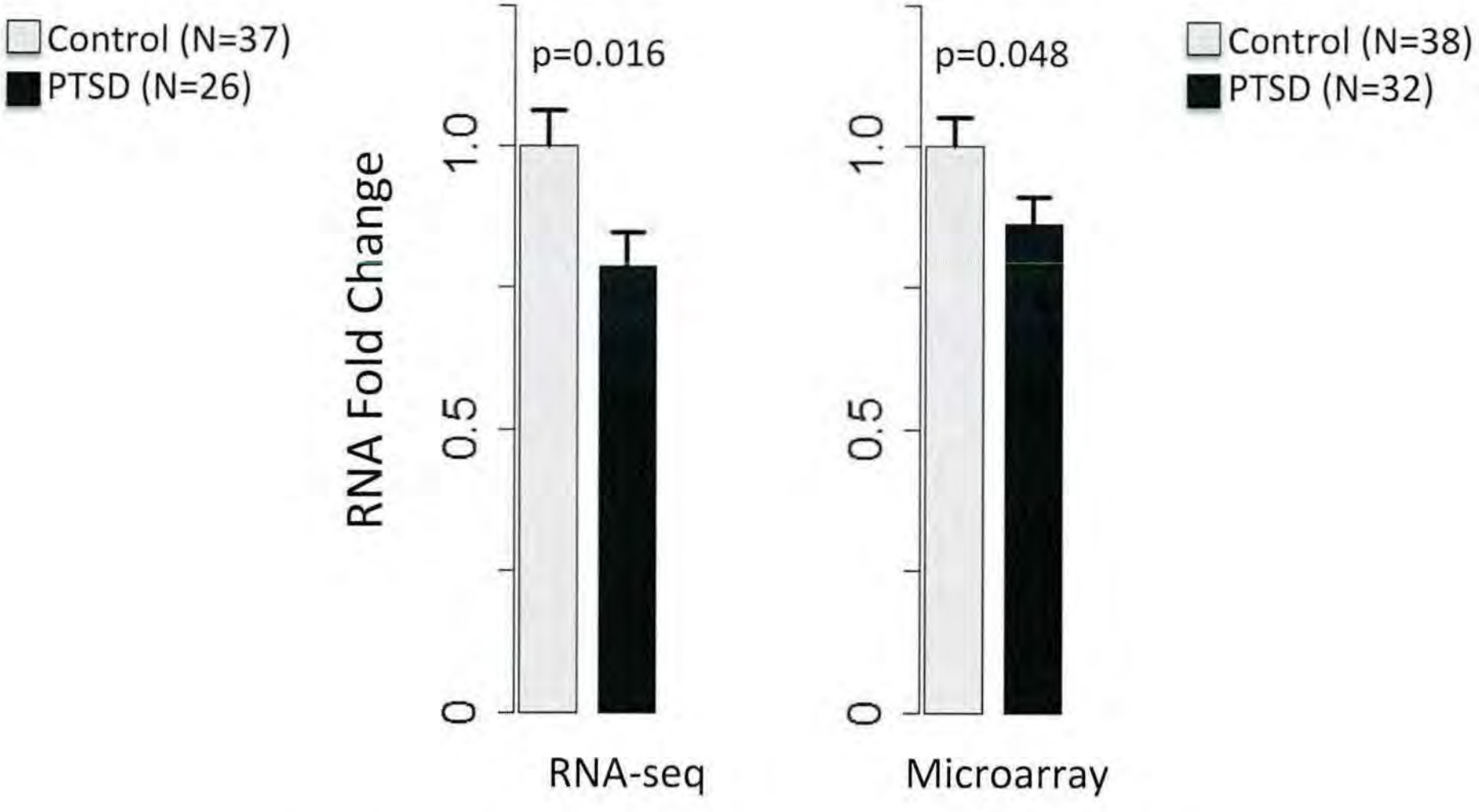
Subjects: 36 PTSD cases, 63 controls
At 6 months post-deployment
Conditioned on pre-deployment methylation and combat exposure

Mean (±SE) levels of methylation in CSMD1 probe is significantly lower in PTSD cases compared to controls three months after exposure to combat



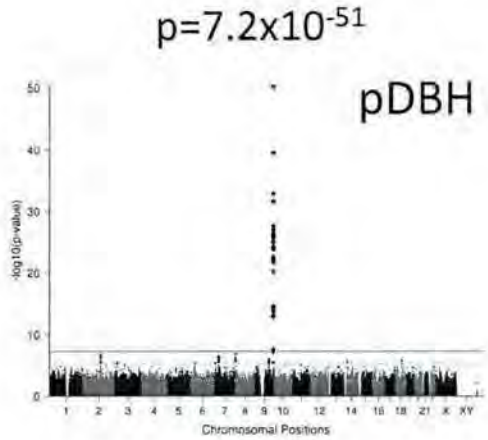
Linear regression (β-regression) with predictors cases status, predeployment methylation, and 3 PC's

Significantly lower gene expression in CSMD1 in PTSD subjects compared to controls at post-deployment assessments



Data represent fold change ($2^{-\Delta VST}$) ± SE, relative to controls, following normalization and VST transformation

Mendelian Randomization in MRS

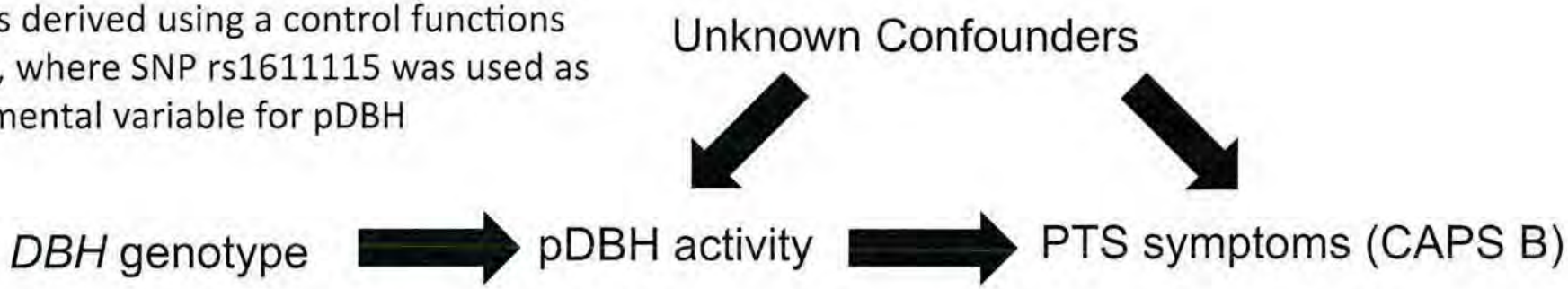


- Dopamine beta-hydroxylase (DBH) catalyzes formation of norepinephrine
- DBH activity was previously found to be associated with PTSD

DBH as a causal predictor of PTSD re-experiencing symptoms:

- pDBH levels and PTSD re-experiencing symptoms were found to be **positively associated** (p = 0.005)
- Top SNP rs1611115 was employed as a genetic instrument to test for a **causal effect** of pDBH on PTSD
- unconfounded estimate of the association of pDBH and re-experiencing symptoms **was significant** (beta=0.26, p = 0.002), indicating causality

MR estimates for the effect of pDBH on PTSD symptoms derived using a control functions approach, where SNP rs1611115 was used as an instrumental variable for pDBH



Mustapic et al. (2014). The catecholamine biosynthetic enzyme dopamine β-hydroxylase(DBH): First genome-wide search positions trait-determining variants acting additively in the proximal promoter. Human Molecular Genetics.

Conclusion

- Genome-wide significant associations of *PRTFDC1* with PTSD
- Polygenic risk scores for bipolar disorder are associated with PTSD
- Genome-wide significant associations of *CSMD1* with PTSD symptom changes after exposure to combat
- Integration of methylome and transcriptome data to support GWAS findings
- MRS is a great resource (e.g. for Mendelian Randomization)

Collaborators and Support

Marine Resiliency Study
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MEG Imaging Markers for Mild TBI and PTSD

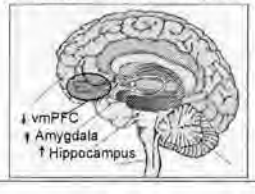
Mingxiong Huang Ph.D.[1,2] and Dewleen G. Baker M.D.[1,2]

[1]VA San Diego Healthcare System, San Diego, CA; [2]University of California, San Diego, CA, USA

Introduction

Traumatic brain injury (TBI) is a leading cause of sustained impairment in military and civilian populations. However, mild TBI (mTBI) can be difficult to detect using conventional MRI or CT. Injured brain tissues in mTBI patients generate abnormal slow-waves (1-4 Hz) that can be measured and localized by resting-state magnetoencephalography (MEG). Post-traumatic stress disorder (PTSD) is another leading cause of sustained impairment, distress, and poor quality of life in military personnel, veterans, and civilians. Indirect functional neuroimaging studies using PET or fMRI with fear-related stimuli support a PTSD neurocircuitry model that includes amygdala, hippocampus, and ventromedial prefrontal cortex (vmPFC). However, it is not clear if this model can fully account for PTSD abnormalities detected directly by electromagnetic-based source imaging techniques (i.e., MEG) in resting-state.

Conventional PTSD Neurocircuitry: hypo-activity in vmPFC, hyper-activity in amygdala and hippocampus



Research Subjects and MEG Exams

Study 1 examined resting-state MEG signals. We develop a voxel-based whole-brain MEG slow-wave imaging approach for detecting abnormality in patients with mTBI on a single-subject basis in 84 mTBI patients with persistent post-concussive symptoms (36 from blasts, and 48 from non-blast causes). A normative database of resting-state MEG source magnitude images (1-4 Hz) from 79 healthy control subjects was established for all brain voxels. The high-resolution MEG source magnitude images were obtained by our Fast-VESTAL method [1].

Study 2 examined resting-state MEG signals in 25 active-duty service members and veterans with PTSD and 30 healthy volunteers. We studied voxel-wise MEG source magnitude images for different frequency bands: alpha (8-12 Hz), beta (15-30 Hz), gamma (30-80 Hz), high-gamma (80-150 Hz), and low-frequency (1-7 Hz) bands [2][3].

Results of MEG Study 1: mTBI

Positive detection rates of abnormal MEG slow-wave source imaging (1-4 Hz):

- ➢0% for normal control subjects.
- ➢86.1% in blast mTBI
- ➢83.3% in non-blast mTBI
- ➢84.5% in combined blast and non-blast mTBI group.

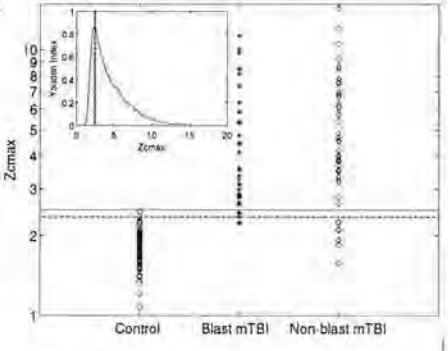


Fig. 1: Z_{cmax} values obtained from MEG source imaging for 1-4 Hz are plotted separately for 1) healthy control, 2) mild blast-induced TBI, and 3) mild non-blast-induced TBI, groups respectively. The embedded plot: the Youden index is plotted as a function of the Z_{cmax} cutoff. The solid and dashed lines in both plots indicate threshold [2].

High sensitivity of MEG slow-wave imaging allows us to detect abnormal slow-wave generations in a single-subject basis as showed in Fig. 2. The results showed that injuries were not homogeneous

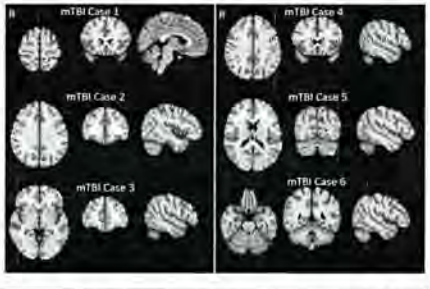


Fig. 2: Single-subject-based analysis showing statistically abnormal MEG source-wave sources in representative mTBI cases. Left column (transverse), middle column (coronal), right column (sagittal).

MEG slow-waves also positively correlated with persistent mTBI symptoms as shown in Fig. 3.

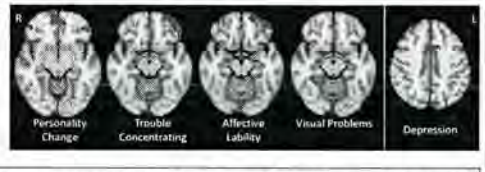


Fig. 3: MEG slow-wave source magnitude significantly correlated with PCS in blast mTBI group (first 4 panels) and non-blast mTBI group (last panel). FDR corrected $p<0.05$.

Results of MEG Study 2: PTSD

In contrast to the healthy volunteers, individuals with PTSD showed: 1) hyperactivity from amygdala, hippocampus, posterolateral orbitofrontal cortex (OFC), dorsomedial prefrontal cortex (dmPFC), and insular cortex in high-frequency (i.e., beta, gamma, and high-gamma) bands (Fig. 4), 2) hypoactivity from vmPFC, Frontal Pole (FP), and dorsolateral prefrontal cortex (dlPFC) in high-frequency bands; 3) extensive hypoactivity from dlPFC, FP, anterior temporal lobes, precuneous cortex, and sensorimotor cortex in alpha and low-frequency bands; 4) in individuals with PTSD, MEG activity in the left amygdala and posterolateral OFC correlated positively with PTSD symptom scores, whereas MEG activity in vmPFC and precuneous correlated negatively with symptom score [2]. Individuals with comorbid PTSD and mTBI showed abnormal MEG delta-waves (1-4 Hz) as evidences of mTBI [3]. The abnormal MEG delta waves generated from vmPFC and dlPFC areas suggest that these areas may be injured in mTBI. The vmPFC and dlPFC regions are also implicated in the PTSD neurocircuitry, thus, abnormal delta-wave findings from the same areas suggests that mTBI to these brain areas may lead to the development of PTSD [3].

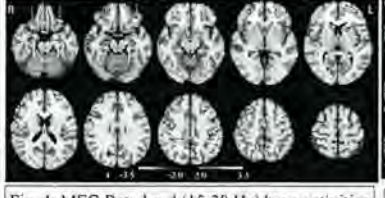


Fig. 4: MEG Beta-band (15-30 Hz) hyperactivities (PTSD>Ctrl, red-hot color) and hypoactivities (PTSD<Ctrl, blue-cold color) in PTSD vs controls
•Hyper-activity: L+R Amygdala (white arrows), L hippocampus, L+R posterolateral OFC (magenta arrows), R insular cortex, PCC (brown arrow).
•Hypo-activity: vmPFC (green arrows), L+R dlPFC, precuneus cortex, L+R frontal poles, L temporal poles, etc.

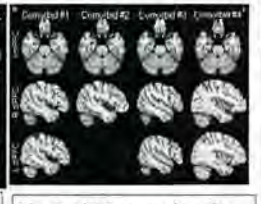


Fig. 5: MEG source imaging showing slow-wave (1-4 Hz) generation in four patients with comorbid mTBI and PTSD from vmPFC and dlPFC suggests that mTBI may potentiate the development of PTSD.

Conclusions

Study 1 provides an effective way for using MEG slow-wave source imaging to localize affected areas and supports MEG as a tool for assisting the diagnosis of mTBI.

Study 2 showed that MEG source imaging technique revealed new abnormalities in the resting-state electromagnetic signals from the PTSD neurocircuitry. Particularly, posterolateral OFC and precuneous may play important roles in the PTSD neurocircuitry model.

References

[1] Huang et al., NeuroImage: Clinical, 5: 109-119, 2014
[2] Huang et al., NeuroImage: Clinical, 5: 408-419, 2014.
[3] Huang, Risling, Baker, Psychoneuroendocrinology. 2015, pii: S0306-4530(15)00058-X. doi: 10.1016/j.psyneuen.2015.02.008.

Autonomic Nervous System and Immune Markers of PTSD Risk and Resilience

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Disclosure

I have no real or apparent conflicts of interest to report.

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Topics

- Marine Resiliency Study & Data set
- Background: Stress, PTSD &
  - Inflammation
  - Autonomic Nervous System
  - Reflex arc
- MRS Findings & Progress to date
- Future directions:
  - Actionable findings
  - Future directions

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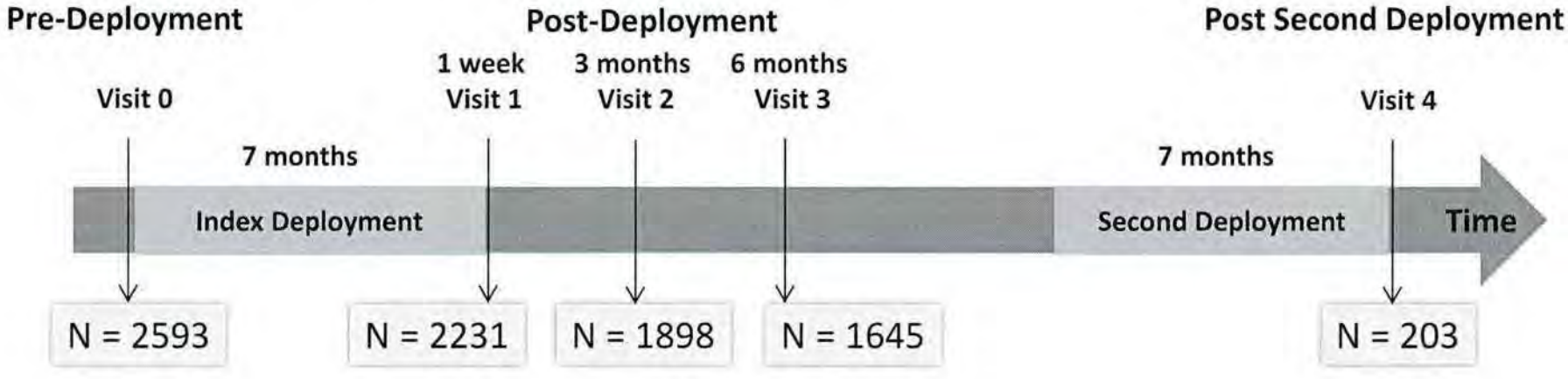
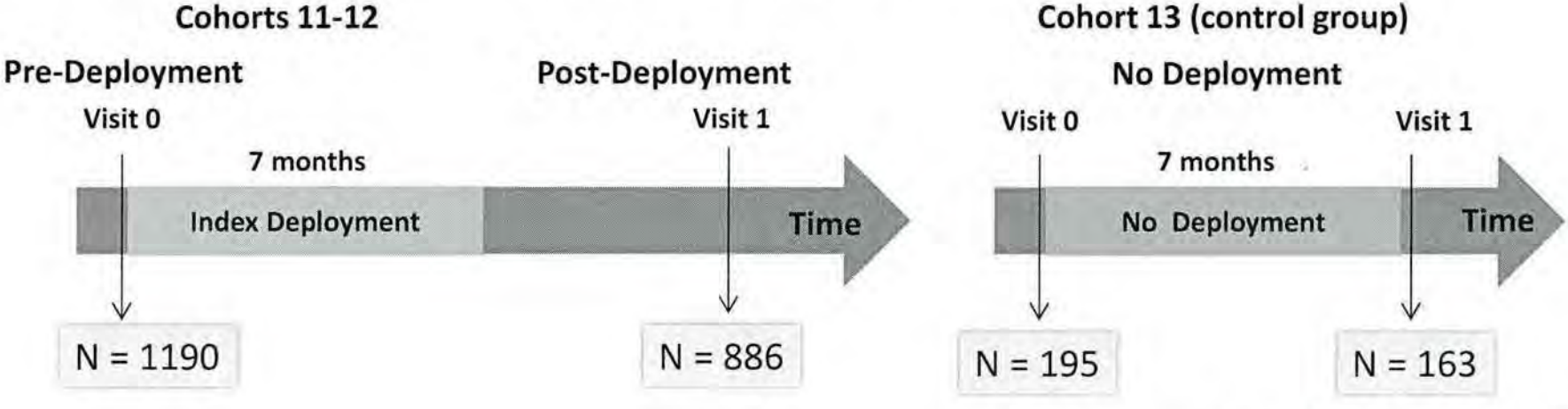
Marine Resiliency Study

MRS: 2008 – 2011 (PIs: Baker, Nash, Litz) Cohorts 1-4



MRSII: 2011 – 2015 (PIs: Baker, Risbrough, Geyer) Cohorts 11-13



Timeline and Enrollment

MRS Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews

Historical
- Self-report questionnaires

Neuropsychological
- Attention, Memory, Executive Function, Reasoning, Social Cognition (AMAM & GUR)

Biological assessments

Biomarkers
- NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- Startle threshold and habituation, fear conditioning and extinction, heart rate variability

Metabolomics

Imaging (DTI/MEG)

MRS Secure database

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Genomics

- **GWAS** (complete data)
- **Methylome** (subset, pre-post)
- **Transcriptome** (subset, pre-post)

DSM-IV PTSD diagnosis using CAPS

• **Criterion A.** The person has been exposed to a traumatic event in which both of the following were present:
(1) the person experienced, witnessed, or was confronted with an event or events that involved actual or threatened death or serious injury, or a threat to the physical integrity of self or others

• **F1/I2 rule: frequency of 1 and intensity of 2** **(range: 0-4 for both)**
experienced it at least once or twice during the last month
distress/discomfort: moderate, distress clearly present but still manageable, some disruption of activities

• **DSM-IV PTSD diagnosis:**

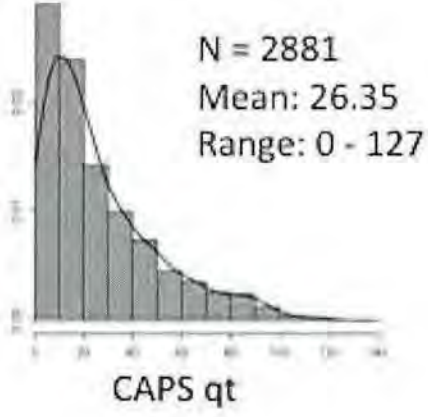
Full: 1B, 3C, 2D

Partial stringent: 1B, 2C, 2D
Partial lenient: 1B, 3C OR 2D

full dx

partial+full

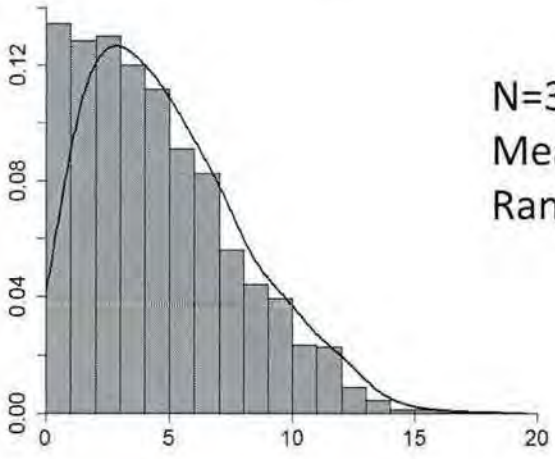
• **Symptom clusters:**
B: Reexperiencing (B1-5)
C: Avoidance / Numbing (C1-7)
D: Hyperarousal (D1-5)



• **CAPS summary score:**
Sum of all frequencies and intensities of the 17 questions (range: 0 – 136)

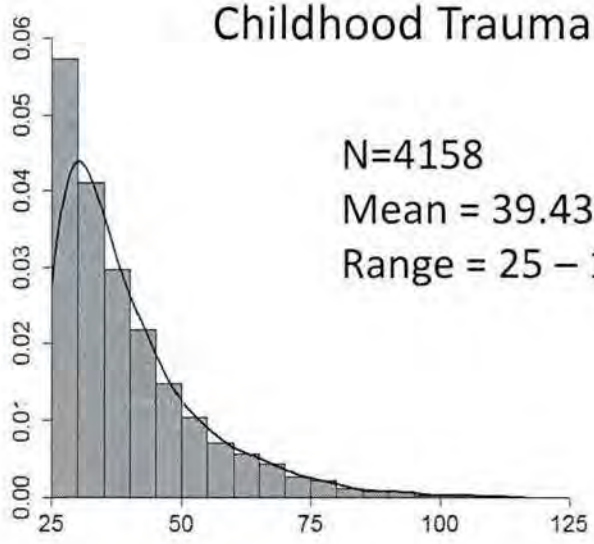
Trauma measures

Adult Trauma (Life Events Checklist LEC)



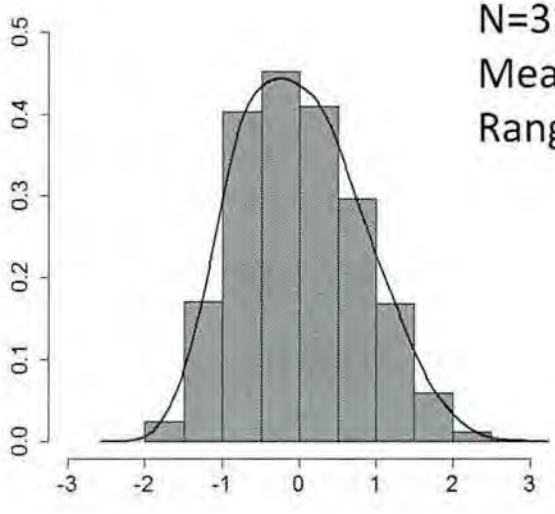
N=3958
Mean = 4.9
Range = 0 - 17

Childhood Trauma (CTQ)



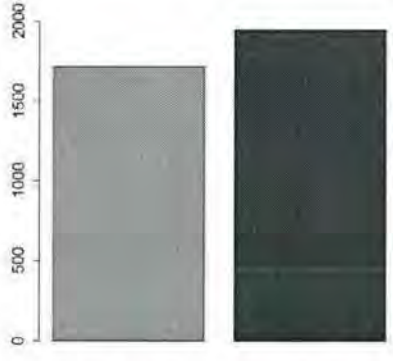
N=4158
Mean = 39.43
Range = 25 – 107.5

Combat Exposure (DRRI Composite)



N=3145
Mean = 0
Range = -1.92 – 2.89

Deployments Prior to Study Enrollment

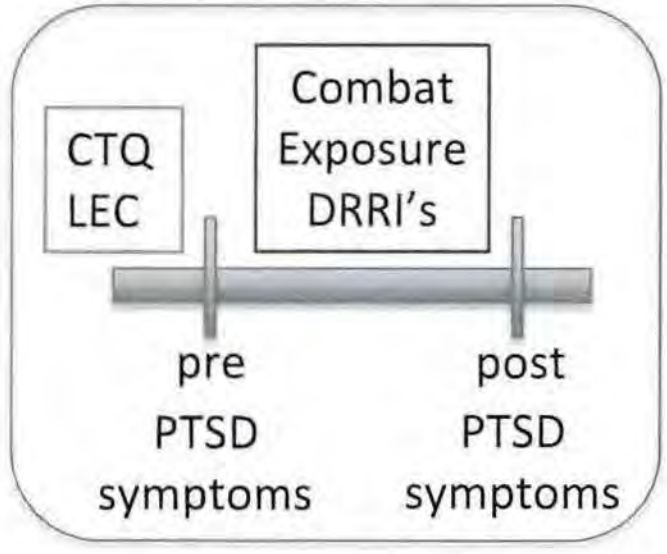


No Prior Deployment

Prior Deployment

53% of subjects had at least one prior deployment

Trauma measures in MRS are highly Predictive of PTSD symptoms (CAPS)



Trauma measures predicting pre-deployment CAPS

Variable	% VE	p	cum % VE	p cum
LEC	1.93%	< 2.2e-16	1.93%	<2e-16
CTQ	1.50%	< 2.2e-16	2.84%	<2e-16
Prior Deployment	0.04%	0.0086	2.84%	0.8372

Trauma measures predicting post-deployment CAPS

Variable	% VE	p	cum % VE	p cum
CAPS pre-depl.	2.80%	< 2.2e-16	2.80%	< 2.2e-16
DRRI	2.70%	< 2.2e-16	5.61%	< 2.2e-16
CTQ	1.19%	< 2.2e-16	5.92%	8.92e-10
LEC	0.74%	< 2.2e-16	6.01%	0.00225

DRRI's: composite score of combat exposure measures
CTQ: Childhood Trauma Questionnaire
LEC: Life Events Checklist at pre-deployment
%VA: % variability explained; cum.: cumulative

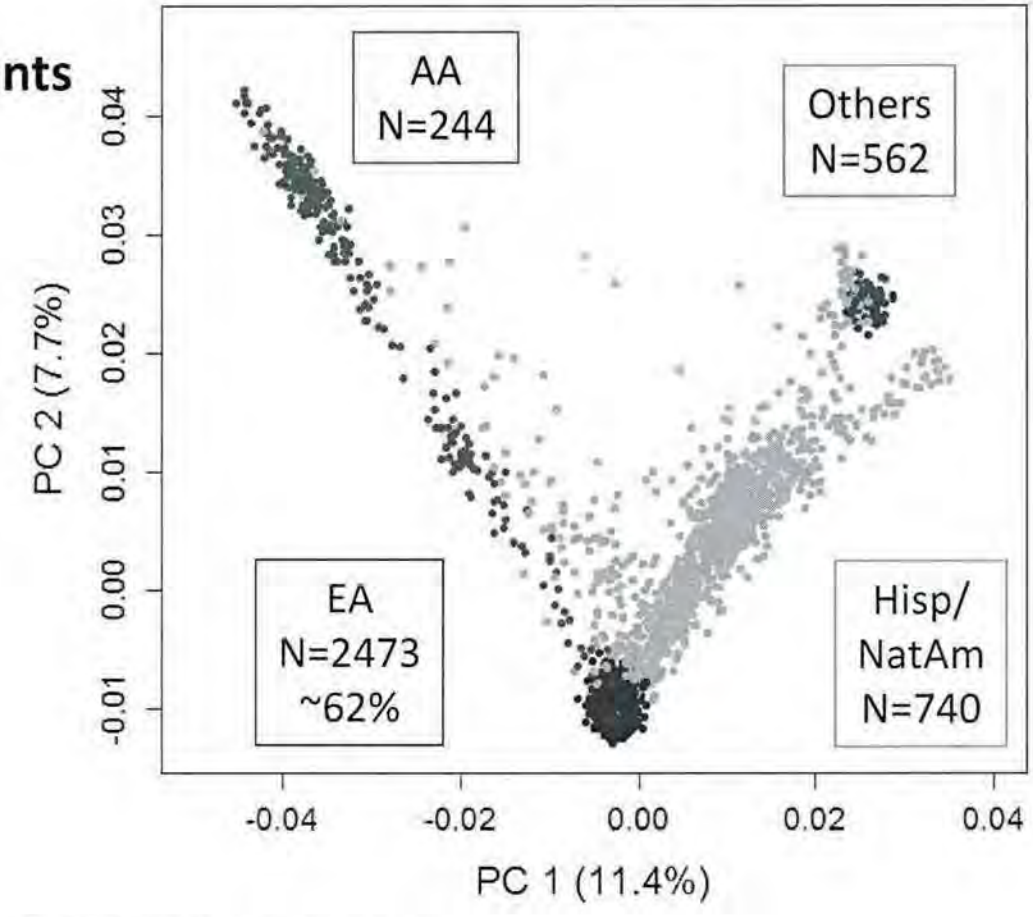
Zero inflated negative binomial regression

Ancestral composition of MRS participants

Ancestry determination:

- based on 1,783 AIMs and HGDP reference subjects

Principal component (PC) analysis of GWAS subjects (N=4019) and reference subjects (N=944) from 52 world populations from the HGDP dataset.



Incorporation of ancestry information into statistical analyses:

- e.g. for genomics, epigenetics, biomarker association analyses
- separate analyses for each of the 4 main ancestry groups, followed by meta-analyses across ancestry groups
- incorporation of AIMs-derived PCs as covariates

AIMs: ancestry-informative markers
HGDP: Human Genome Diversity Panel
GWAS: Genome-wide association study

MRS Resources and Progress Overview

Psychological , Behavioral and Physiological assessments

Mental and Physical Health
- Clinical interviews and self report: 7 papers published

Psychophysiology and Neurocognition
- Hearing/Tinnitus, Startle, HRV, Neurocognitive Battery: 5 papers published

MRS Secure database

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

Biobank

Ongoing requests for samples to be used for replication studies and new grant proposals

Biological assessments

Biomarkers
- Immune, hormone and metabolomic predictors of risk : 3 papers published

Imaging
- **MEG, MRI, DTI**: 4 papers published
- **TBI/Tinnitus Imaging Study** (funded)
- **In-country Blast monitoring with follow up Imaging** (funded)

Genomics

- **MRS GWAS R01** (funded)
- **PGC PTSD consortium** (funding likely)
- **International HRV GWAS consortium**
- **PGC PTSD EWAS consortium** (funding likely)
- **Transcriptome**: 3 papers published; proposals submitted

Neuropharmacology 62 (2012) 663–673

Contents lists available at SciVerse ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Invited review

Biomarkers of PTSD: Neuropeptides and immune signaling

Dewleen G. Baker[a,b,*], Caroline M. Nievergelt[b], Daniel T. O'Connor[c]

- Psychological stress & trauma are associated with inflammation
  (Segerstrom & Miller, 2004 for review; Pariante et al 2007; Carpenter et al 2010; Kiecolt-Glaser et al 2011; Neylan et al 2012; O'Donovan et al 2012)
- PTSD is associated with increased inflammation and confers a higher risk for autoimmune disorders
  (Cytokines: Hoge et al 2009; Canetti et al 2014; Newton et al 2014; Baker et al 2009 for review & C-Reactive Protein: Spitzer et al 2010; Heath et al 2013; Plantinga et al 2013; O'Donovan et al 2015)

RAPID PUBLICATION

AMERICAN JOURNAL OF
medical genetics
Neuropsychiatric Genetics

Blood-Based Gene-Expression Predictors of PTSD Risk and Resilience Among Deployed Marines: A Pilot Study

Stephen J. Glatt,[1*] Daniel S. Tylee,[1] Sharon D. Chandler,[2] Joel Pazol,[2] Caroline M. Nievergelt,[3,4,5] Christopher H. Woelk,[4,6] Dewleen G. Baker,[3,4,5] James B. Lohr,[3,4,5] William S. Kremen,[2,3,5] Brett T. Litz,[7] Ming T. Tsuang,[2,3,4,5,8,9,10] and Marine Resiliency Study Investigators

- Using MRS longitudinal data, we have a number of papers analyzing gene expression between PTSD cases and controls
- For several genes, gene expression was dysregulated in PTSD compared to controls
- A disproportionate number of these genes involved cellular immunity

Is Inflammation a risk factor for PTSD?

Research

JAMA Psychiatry

Formerly *Archives of General Psychiatry*

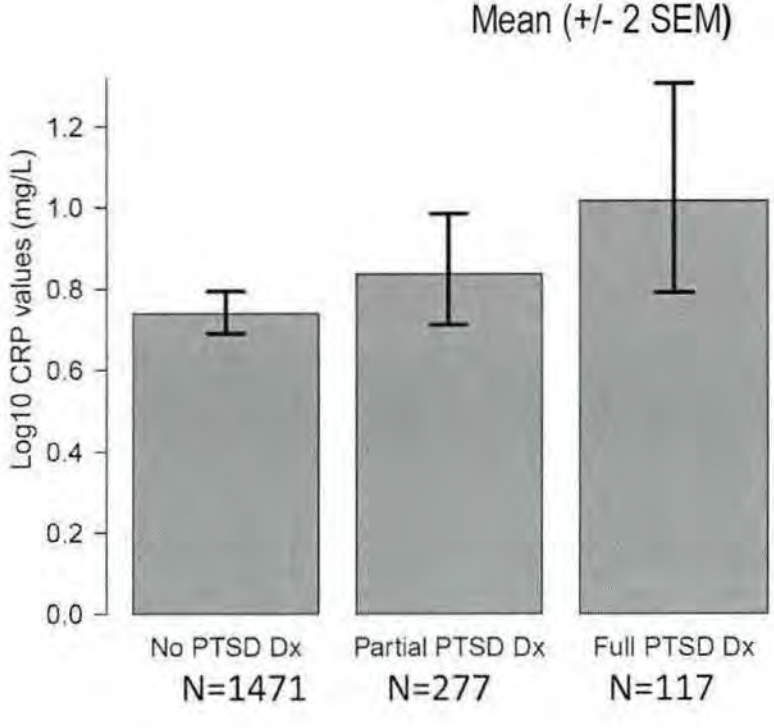
Original Investigation

Assessment of Plasma C-Reactive Protein as a Biomarker of Posttraumatic Stress Disorder Risk

Satish A. Eraly, MD, PhD; Caroline M. Nievergelt, PhD; Adam X. Maihofer, MS; Donald A. Barkauskas, PhD; Nilima Biswas, PhD; Agorastos Agorastos, MD; Daniel T. O'Connor, MD; Dewleen G. Baker, MD; for the Marine Resiliency Study Team

CRP measured at pre-deployment is significantly predicting PTSD symptoms post-deployment

Parameter	OR	95% CI	P
Cohort 2	0.846	0.537 - 1.347	0.475
Cohort 3	0.514	0.314 - 0.845	0.008
Cohort 4	0.923	0.552 - 1.553	0.762
CAPS0	1.053	1.045 - 1.062	0
CES	1.035	1.019 - 1.051	0
PBE	1.103	1.058 - 1.151	0
log CRP	1.3	1.026 - 1.646	0.029



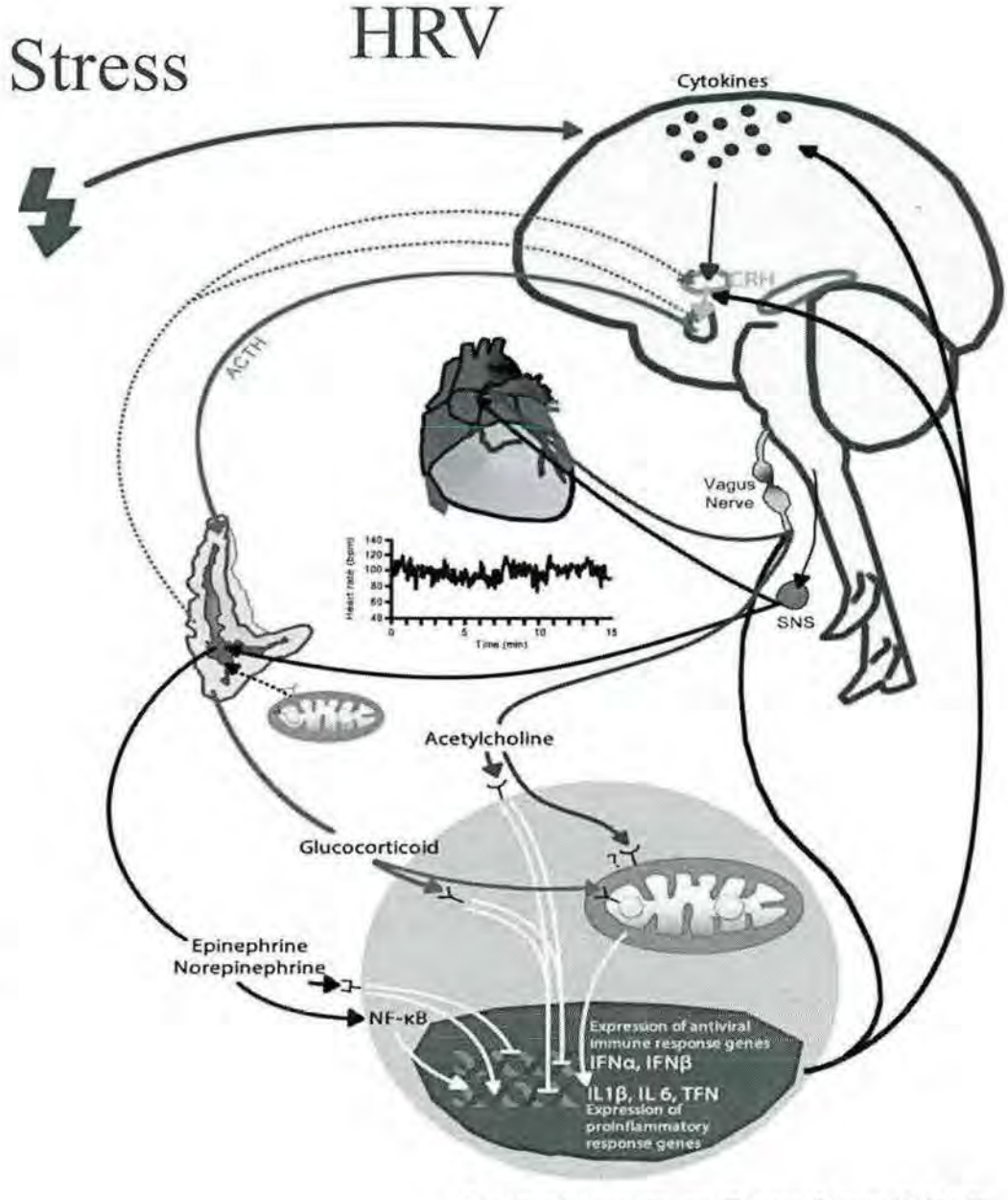
Vagal tone as a moderator of inflammation

Behavioral considerations:

- Body mass index
- Sleep
- Alcohol Use
- Tobacco Use

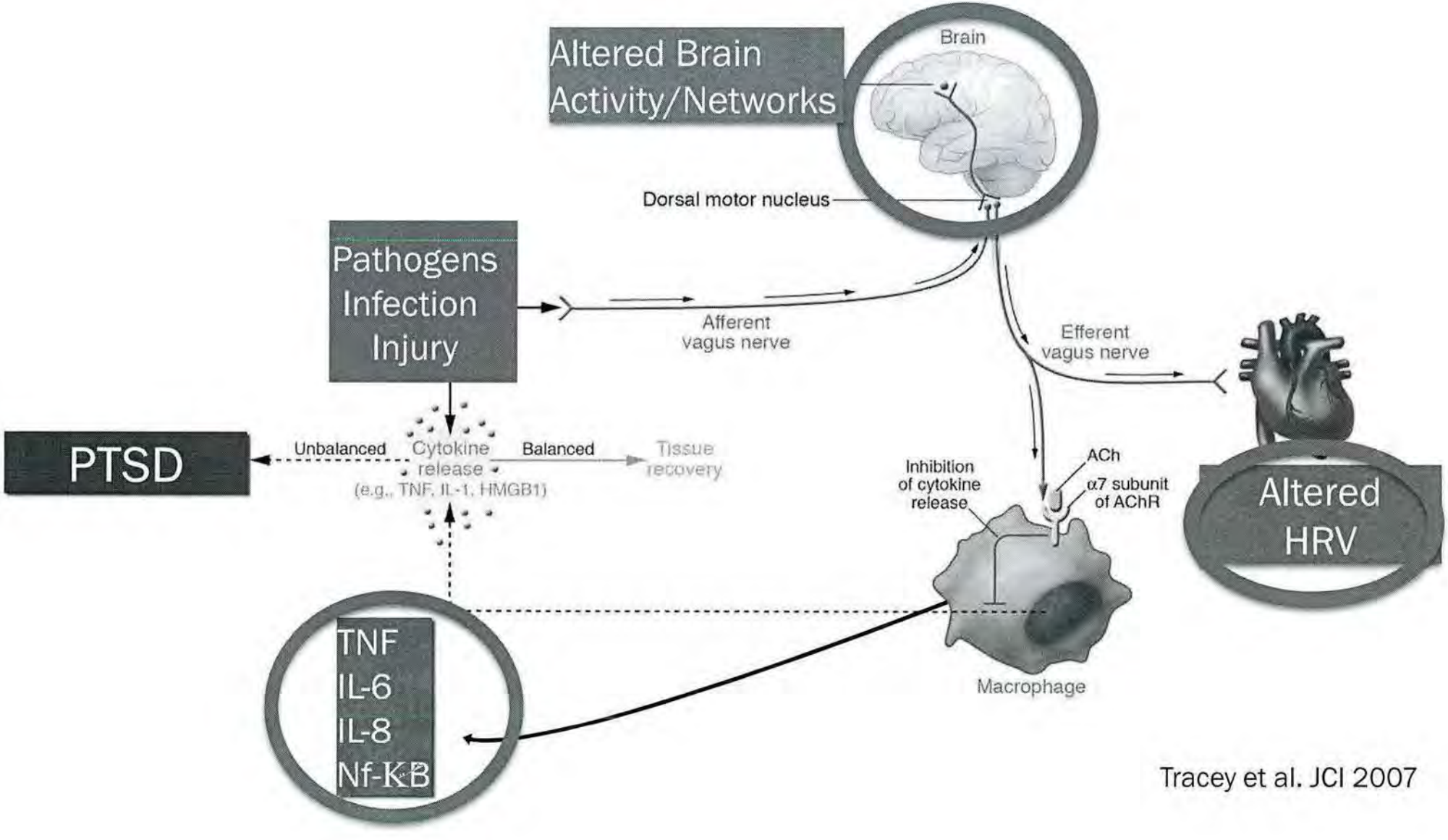
Biological determinants:

- HPA axis (cortisol)
- Sympathetic outflow
- Parasympathetic (vagal) tone



Sturmburg et al.Front Phys 2015

Vagus Nerve, Inflammatory Reflex, and Autonomic Tone



Tracey et al. JCI 2007

Is Vagal Tone (HRV) associated with PTSD?

HRV, determined by fluctuation of heart rate, measured in time, frequency or non-linear domains, is a powerful index of autonomic function and an indicator of cardiac risk

(Agorastos et al, Diminished Vagal and/or Increased Sympathetic Activity in Post-Traumatic Stress Disorder, Comprehensive Guide to Post-Traumatic Stress Disorder, Springer International Publishing, Switzerland 2015 for review)

Accumulating evidence in PTSD:

- Diminished HRV has been observed in PTSD
  (Cohen et al 1997, 2000; Haley et al.2004; Mellman et al 2004; Jovanovic et al 2009; Ginsberg et al 2010; Song et al 2011; Tan et al 2011; Agorastos et al 2013)
- PTSD patients show blunted diurnal variation (reduced HR and HRV increase) at night
  (Cohen et al 1997; Haley et al. 2004; Bedi and Arora 2007; Pole 2007; Mellman et al. 2009; Woodward et al. 2009; Agorastos et al. 2013)

The Official Journal of the American Psychosomatic Society
PSYCHOSOMATIC MEDICINE
Journal of Biobehavioral Medicine

Heart Rate Variability Characteristics in a Large Group of Active-Duty Marines and Relationship to Posttraumatic Stress

Minassian, Arpi PhD; Geyer, Mark A. PhD; Baker, Dewleen G. MD; Nievergelt, Caroline M. PhD; O'Connor, Daniel T. MD; Risbrough, Victoria B. PhD; for the Marine Resiliency Study Team

- Cross-sectional HRV analysis of MRS at pre-deployment supports associations between PTSD and reduced HRV, while accounting for TBI and depression

Is Heart Rate Variability a factor in PTSD Risk?

A Prospective Analysis
Is Heart Rate Variability a Risk Factor for PTSD?

Meta-Analysis of MRS-I and MRS-II pre-deployment LF:HF ratio predicting DSM-IV based PTSD diagnosis 6 months after deployment

Parameter	OR	95% CI	P
DRRI post-deployment	2.84	2.15-3.74	<0.001
LF: HF ratio	1.47	1.10-1.98	0.01

Regression included combat exposure as measured by DRRI. LF:HF ratio was log-transformed.

Original Investigation

Association of predeployment heart rate variability with risk of postdeployment Postraumatic Stress Disorder in active-duty Marines

Arpi Minassian, Ph.D., Adam X. Maihofer, M.S., Dewleen G. Baker, M.D., Caroline Nievergelt, Ph.D., Mark A. Geyer, Ph.D., and Victoria B. Risbrough, Ph.D., for the Marine Resiliency Study Team

JAMA Psychiatry in press.

Actionable Results

Psychological , Behavioral and Physiological assessments

Mental and Physical Health
- Deployment is TBI a strong predictor of PTSD

Psychophysiology and Neurocognition
- **Pre-deployment HRV predicts PTSD**
- Sensorimotor gating may be *trainable* resiliency factor for PTSD

MRS
Secure database

Career History Archival Medical and Personnel System database

As available , use of VA and military service database information in follow-up data collection and analysis

Biobank

Active replication of GWAS and EWAS study findings in collaboration with PRISMO, Army STARRS, Grady Trauma Cohort

Biological assessments

Biomarkers
- **Pre-deployment immune activation state predicts PTSD**

Imaging
- Development of diagnostic MEG signatures for mTBI

Genomics

Transcriptome
- **Pre-deployment gene expression signatures of PTSD Risk**

Genome
- Identified 2 novel genes associated with PTSD risk

Future Directions

- Refinement of MRS immune system variables data collection for more detailed information about vagal-immune system interactions in PTSD and mTBI
  (Baker et al, CDMRP FY 2016-2019)

- Closed-loop transcutaneous vagal nerve stimulator for
  - Physiology challenge studies with imaging
  - Immune system variable collection
  - Studies on prevention and treatment of PTSD

(Lerman et al. NARSAD grant FY 2016-2017)

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- Robert Naviaux, M.D. Ph.D., UCSD
- Richard Hauger, M.D. UCSD/CESAMH
- Mingxiong Huang, M.D., UCSD, VA
- MRS Research Team

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- Kate Yurgil Ph.D., UCSD

Statistics

- Adam Maihofer M.S., UCSD
- Don Barkauskas Ph.D. USC

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- **James Churchill Ph.D. NIMH**

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Thanks also to CESAMH and to NC COSC for Assistance in Data Collection

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Seeking Risk and Resilience Factors for PTSD: The Marine Resiliency Study

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Professor, Department of Psychiatry
University of California San Diego

UCSD

Epidemiology of Psychiatric Disorders

Disorder	US Prevalence (%) Lifetime	12-Month
Any psychiatric disorder	48.0	29.5
Any affective disorder	19.3	11.3
Major depressive disorder	17.1	10.3
Dysthymia	6.4	2.5
Manic episode	1.6	1.3
Any anxiety disorder	**24.9**	**17.2**
Social phobia	12.1	7.1
Simple phobia	11.3	8.8
Generalized anxiety disorder	5.1	3.1
Panic disorder	4.0	2.3
Obsessive-Compulsive Disorder	2.5	2.1
Post-Traumatic Stress Disorder	7.8	3.6
Any substance abuse/dependence	26.6	11.3
Alcohol abuse w/o dependence	9.4	2.5
Alcohol dependence	14.1	7.2

(Kessler RC et al, 1994; Narrow et al, 2002; Ruscio et al, 2007)

DSM-V Trauma/Stress Disorders

- Reactive Attachment Disorder (Infants/Children)
- Disinhibited Social Engagement Disorder
- Post-traumatic Stress Disorder
- Acute stress disorder
- Adjustment Disorders
- Other Specified Trauma- and Stressor-Related Disorder

Trauma/Stress Disorders

DSM-V Diagnosis – Trauma/Stressor Criteria

Criteria apply to adults, adolescents, children older than 6 years

- Exposure to actual or threatened death, serious injury, or sexual violation in one (or more) of the following ways:
  - Directly experiencing the traumatic event(s).
  - Witnessing, in person, the event(s) as it occurred to others.
  - Learning that the event(s) occurred to a close family member or close friend. **Note:** In cases of actual or threatened death of a family member or friend, the event(s) must have been violent or accidental.

Trauma/Stress Disorders

DSM-V Diagnosis – Trauma/Stressor Criteria

– Experiencing repeated or extreme exposure to aversive details of the traumatic event(s) (e.g., first responders collecting human remains, police officers repeatedly exposed to details of child abuse).

  - **Note:** This does not apply to exposure through electronic media, television, movies, or pictures, unless this exposure is work related.

Posttraumatic Stress Disorder

DSM-V Diagnosis

- Presence of one (or more) **intrusion symptoms** associated with the traumatic event(s), beginning after the traumatic event(s) occurred: (re-experiencing)
- Persistent **avoidance** of stimuli associated with the traumatic event(s), beginning after the traumatic event(s) occurred, as evidenced by one or both of the following: avoidance of thoughts/feelings, avoidance of external reminders
- ***Negative alterations in cognitions and mood*** associated with the traumatic event(s), beginning or worsening after the traumatic event(s) occurred, as evidenced by two (or more) of the following: difficulty remembering, *exaggerated negative beliefs/expectations, distorted cognitions*, anhedonia, etc.
- Marked **alterations in arousal and reactivity** associated with the traumatic event(s), beginning or worsening after the traumatic event(s) occurred, as evidenced by two (or more) of the following: irritable behavior and angry outbursts, *reckless or self destructive behavior*, hypervigilance, etc.
- Duration of the disturbance (Criteria B, C, D, and E) is more than 1 month.

Posttraumatic Stress Disorder

DSM-V Diagnostic Features

- **Prevalence among recently exposed populations varies by nature of the event and the context within which it is assessed**

- **Common rates in the US**
  - Projected lifetime risk (under DSM-IV criteria) at age 75 is 8.7% for the general population

  - Reported risk for the Iraq/Afghanistan combatants varies by the publication source, ranging from 5% to 20%
    (Ramachand et al., (2010) Journal of Traumatic Stress 23 (1), 20-68. 2010)

Posttraumatic Stress Disorder

PTSD confers higher risk for a number of Medical Conditions

- PTSD is associated an increased risk for:
  - Atherosclerotic cardiovascular disease
  - Inflammatory and autoimmune disorders
  - Metabolic syndrome
  - Dementia

Historical Background

Post-Vietnam to the Iraq/Afghanistan Wars
Cross-sectional Areas of Research

- PTSD risk/resilience factors (psychosocial)
  - Childhood adversity
  - Level of (index) trauma burden
  - Neurological intactness/IQ
  - Social support

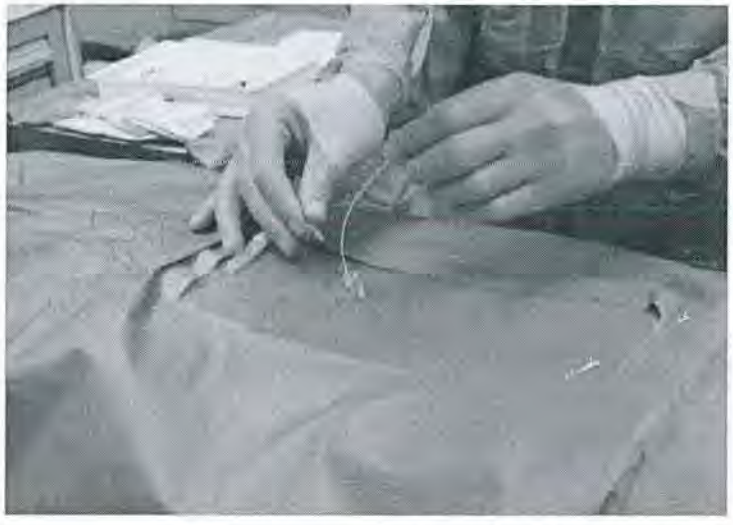
- PTSD – biological system abnormalities

Historical Background

Cross-sectional Research
Stress and Immune Physiology

- In individuals with a diagnosis of PTSD, peripheral and Cerebrospinal fluid studies give evidence for abnormalities associated with:

- The central nervous system (hyperarousal)
- HPA axis
- Autonomic nervous system
- Inflammatory markers



Historical Background

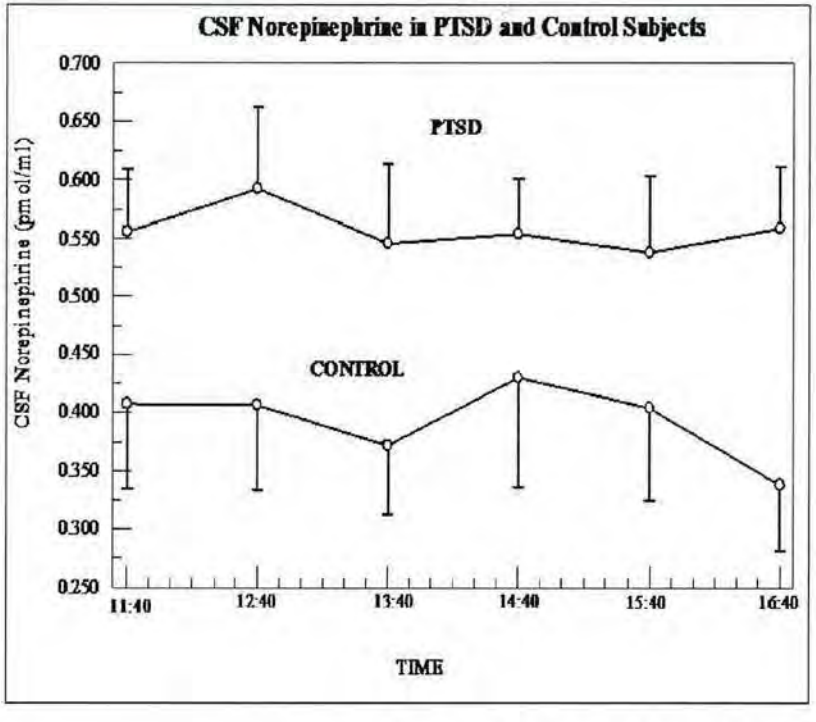
Stress and Immune Physiology

- **Serial cerebral spinal fluid study**
  - » Recruitment of healthy individuals
    - Non-smokers
    - No past alcohol dependence and no current abuse
    - No medications or wash-out for at least 5 half-lives prior to procedure
  - » Standardized Diet for duration of Study – set meals/calories
  - » Controlled environment – no radio, television
  - » Subarachnoid catheter placement at 8AM – 3 hours of rest prior to CSF sampling
  - » 24 (every hour) basal samples of CSF and plasma (11AM to 11AM) assayed for NPY concentration or measured using direct radioimmunoassay or for cortisol concentration

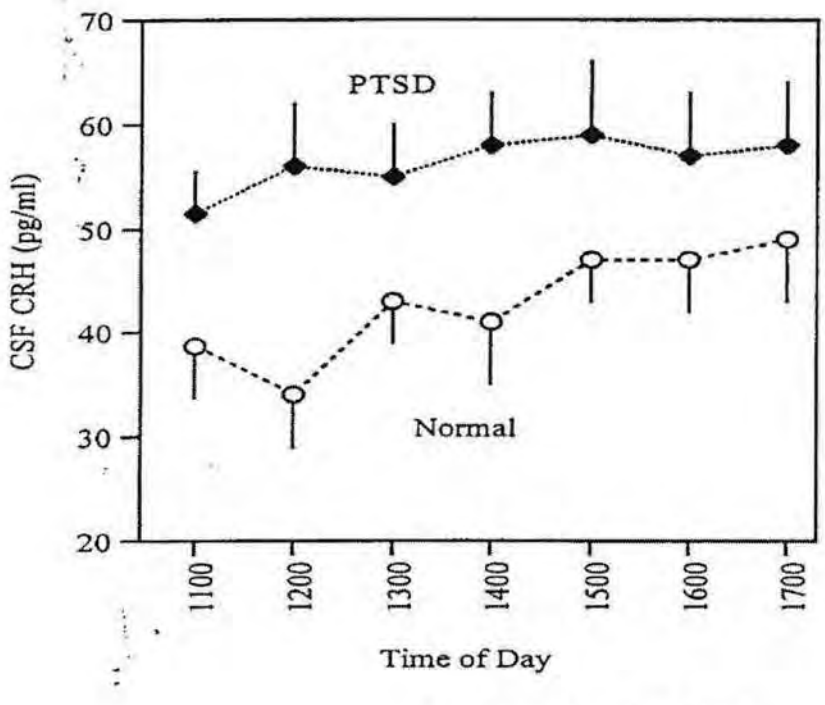
Historical Background

Stress and Immune Physiology

Increased CNS NE and CRF



Geracioti et al., 2001

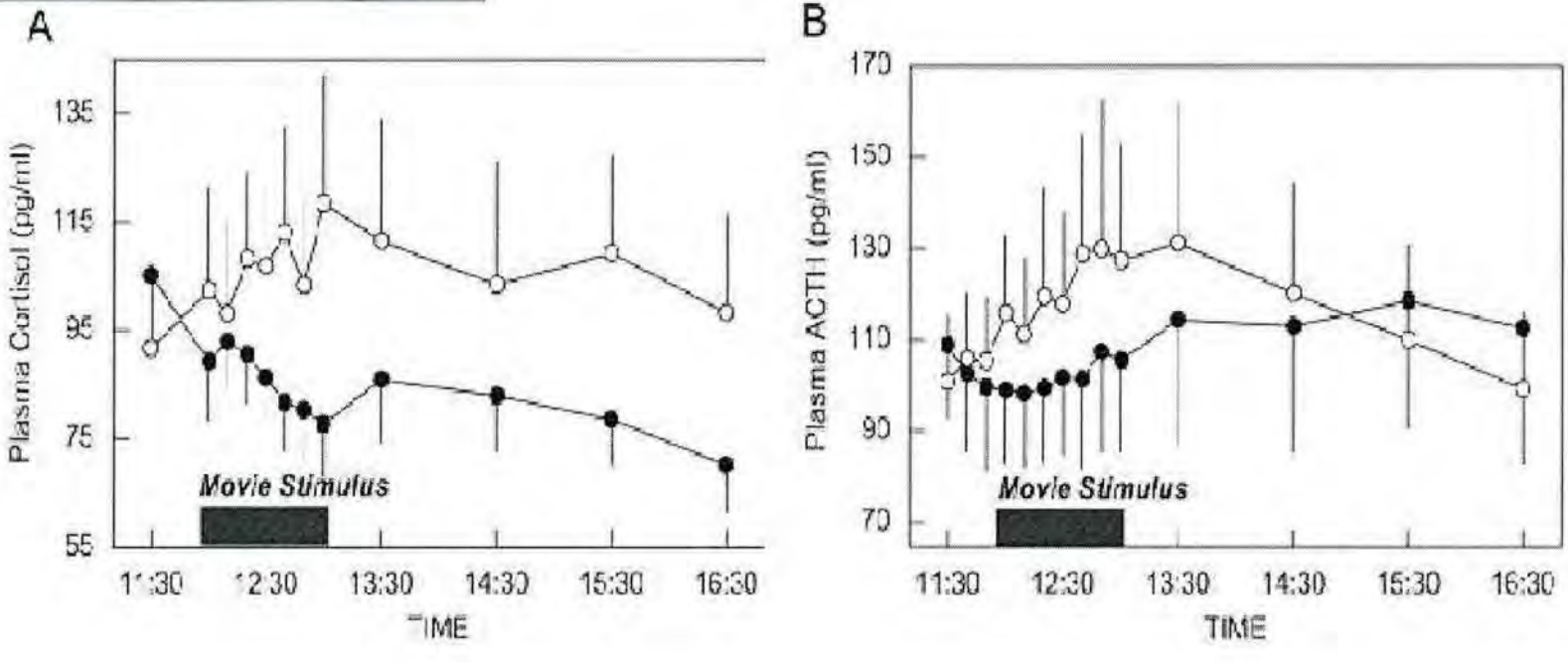


Baker et al., 1999

Historical Background

Stress and Immune Physiology

Abnormal HPA response to stress



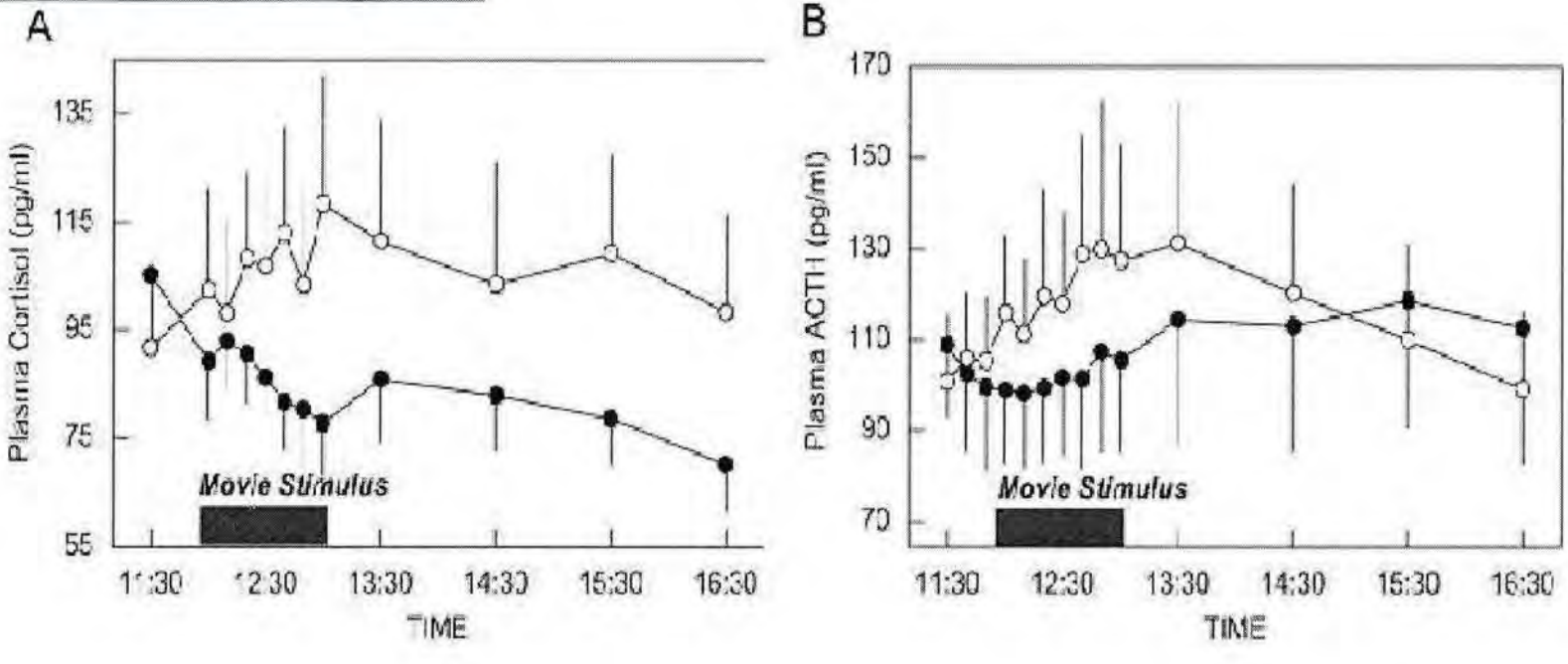
Following Trauma-related (vs neutral) video:

- Significant decline in CSF CRF levels following trauma-related video
- Significant decline in Plasma cortisol levels following trauma video

Geracioti et al., 2001

Historical Background

Stress and Immune Physiology
Abnormal HPA response to stress



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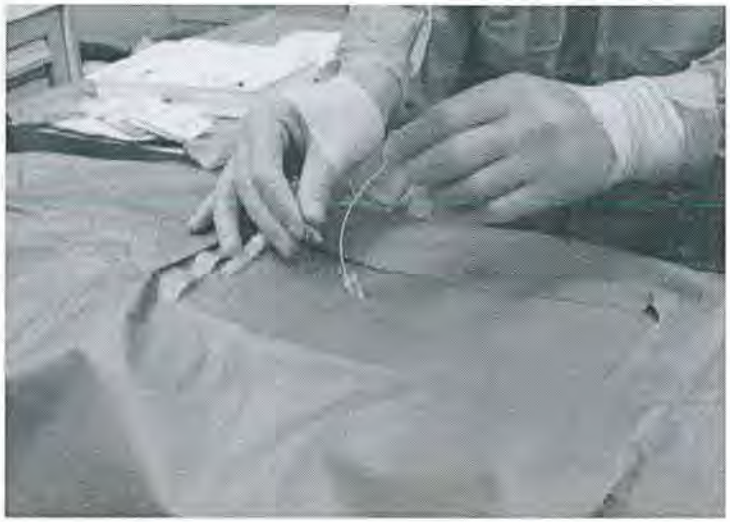
Geracioti et al., 2001



Historical Background

Stress and Immune Physiology Abnormal Autonomic Response to stress & increasing evidence for chronic inflammation

Stress, 2012; Early Online: 1–11

ISSN 1025-3890 print/ISSN 1607-8888 online
DOI: 10.3109/10253890.2012.751369

informa
healthcare

Diminished vagal activity and blunted diurnal variation of heart rate dynamics in posttraumatic stress disorder

AGORASTOS AGORASTOS[1,2], JUDITH A. BOEL[3], PIA S. HEPPNER[4,5], TORBEN HAGER[3], TOBIAS MOELLER-BERTRAM[1,4,5], UZAIR HAJI[5], ARAME MOTAZEDI[5], MATTHEW A. YANAGI[1], DEWLEEN G. BAKER[1,4,5,*], & OLIVER STIEDL[3,*]

[1]*Veterans Affairs Center of Excellence for Stress and Mental Health, VA San Diego, CA, USA,* [2]*Department of Psychiatry and Psychotherapy, University Medical Center Hamburg-Eppendorf, Hamburg, Germany,* [3]*Behavioral Neuroscience Group, Departments of Functional Genomics and Molecular and Cellular Neurobiology, Center for Neurogenomics and Cognitive Research, Neuroscience Campus Amsterdam, VU University, Amsterdam, The Netherlands,* [4]*Department of Psychiatry, University of California, San Diego, CA, USA, and* [5]*VA San Diego Healthcare System, San Diego, CA, USA*

(*Received 14 June 2012; accepted 12 November 2012*)

Marine Resiliency Study

Dewleen G. Baker M.D.

Caroline Nievergelt Ph.D.

Victoria Risbrough Ph.D.

Mark Geyer Ph.D.

UCSD

Marine Resiliency Study

Field Study: 1st Marine Division

- Infantry Battalions, Combat Engineers
- Explosive Ordinance Device (EOD)

Participants: Marines, Navy Personnel

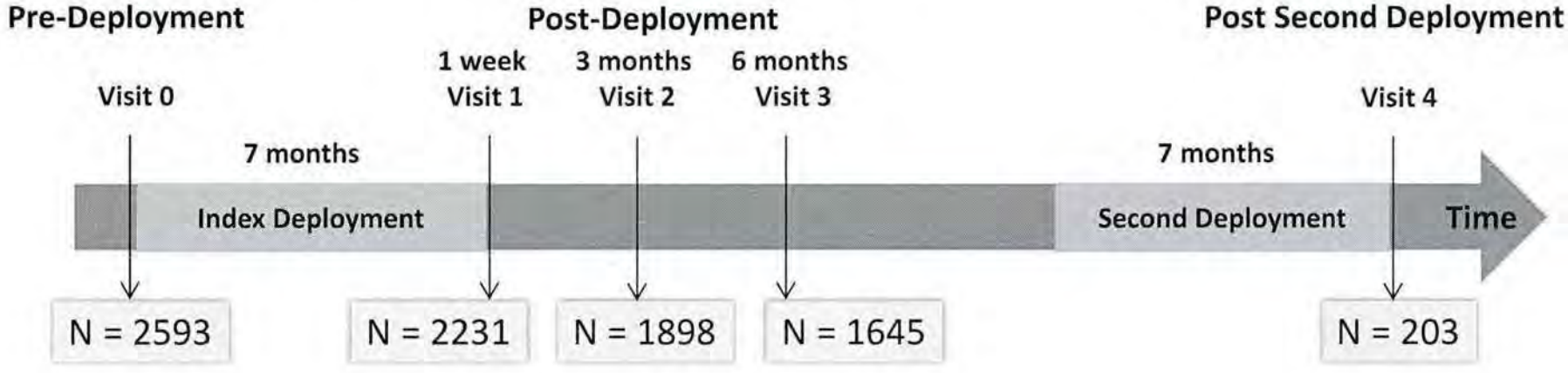
Six-wide semi-permanent data collection trailer at MCAGCC 29 Palms

Setting

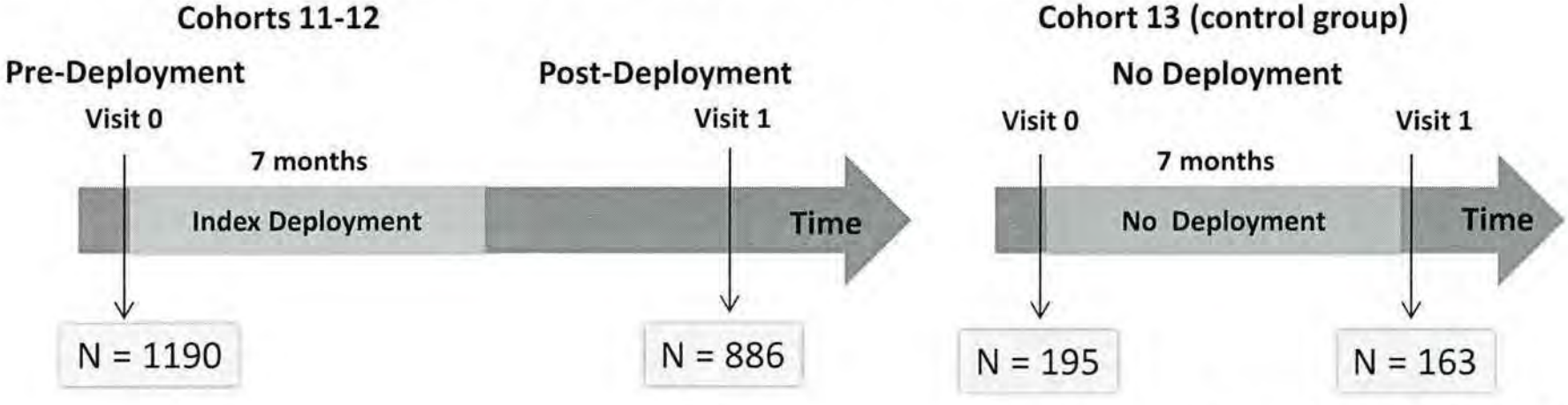
- **Marine Corps Air Ground Combat Center - 29 Palms**
- **Camp Pendleton**

Marine Resiliency Study

MRS: 2008 – 2011 (PIs: Baker, Nash, Litz) Cohorts 1-4



MRSII: 2011 – 2015 (PIs: Baker, Risbrough, Geyer) Cohorts 11-13



Timeline and Enrollment

MRS Longitudinal Data Sources

Psychological and Behavioral assessments

Psychiatric and medical
- Clinical interviews: PTSD/TBI

Historical
- Self-report questionnaires

Neuropsychological
- ANAM + Penn Battery

Career History Archival Medical and Personnel System database

Military archives
- Medical diagnoses
- Hospitalizations
- Outpatient healthcare visits
- Duty status
- Separation date and reason

MRS Secure database

Biological assessments

Biomarkers
- e.g. NPY, CRP, Alpha-amylase, Catecholamines, Cortisol

Hemodynamics
- Pulse and blood pressure

Psychophysiology
- EMG, PPG

Metabolomics

Imaging (MEG/DTI)

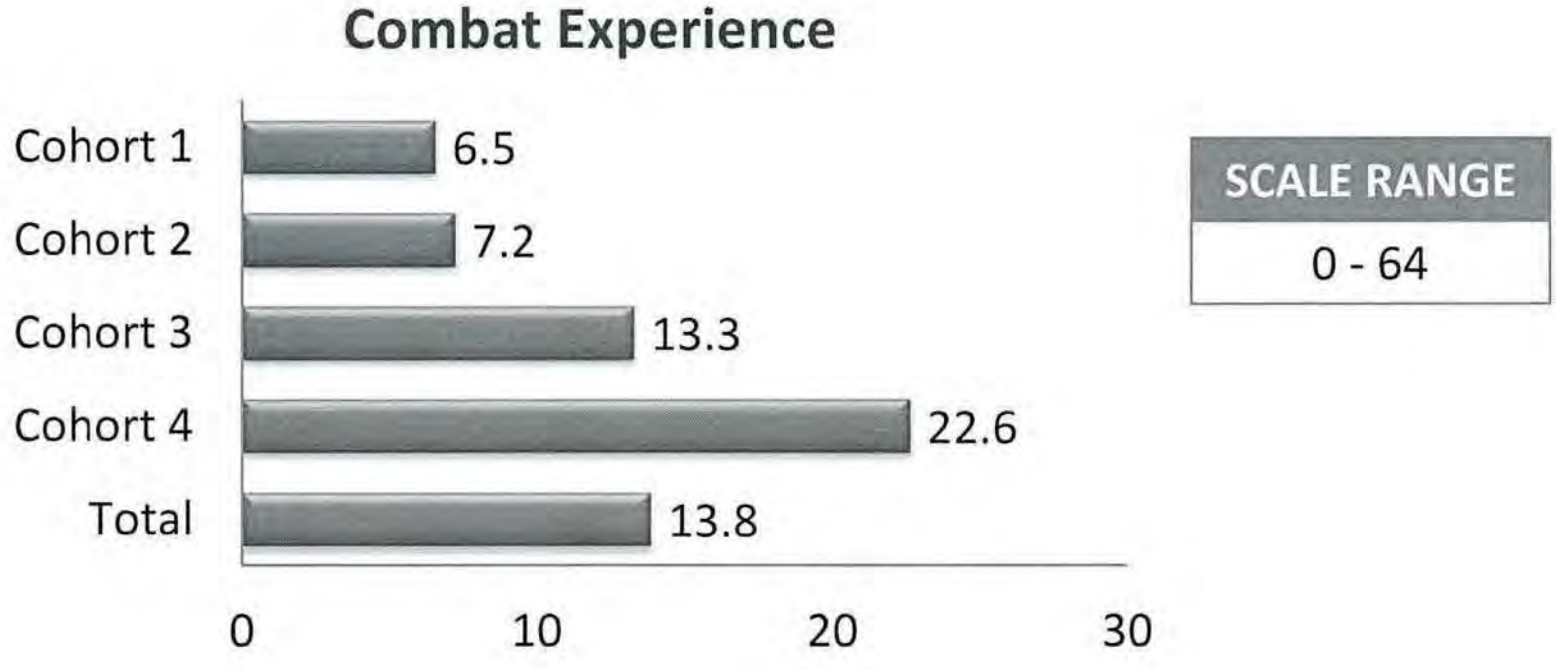
Biobank

Biological samples
- Blood (whole blood, plasma)
- Saliva
- Urine
- DNA / RNA

Genomics*
- **GWAS** (complete data)
- **Methylome** (subset, pre-post)
- **Transcriptome** (subset, pre-post)

***GWAS UCSD,NIH R0-1 Caroline Nievergelt PI**
***Gene expression, UCSD, R21 Ming Tsuang PI**
***Methylome, RNA-seq C Nievergelt MRS-II**

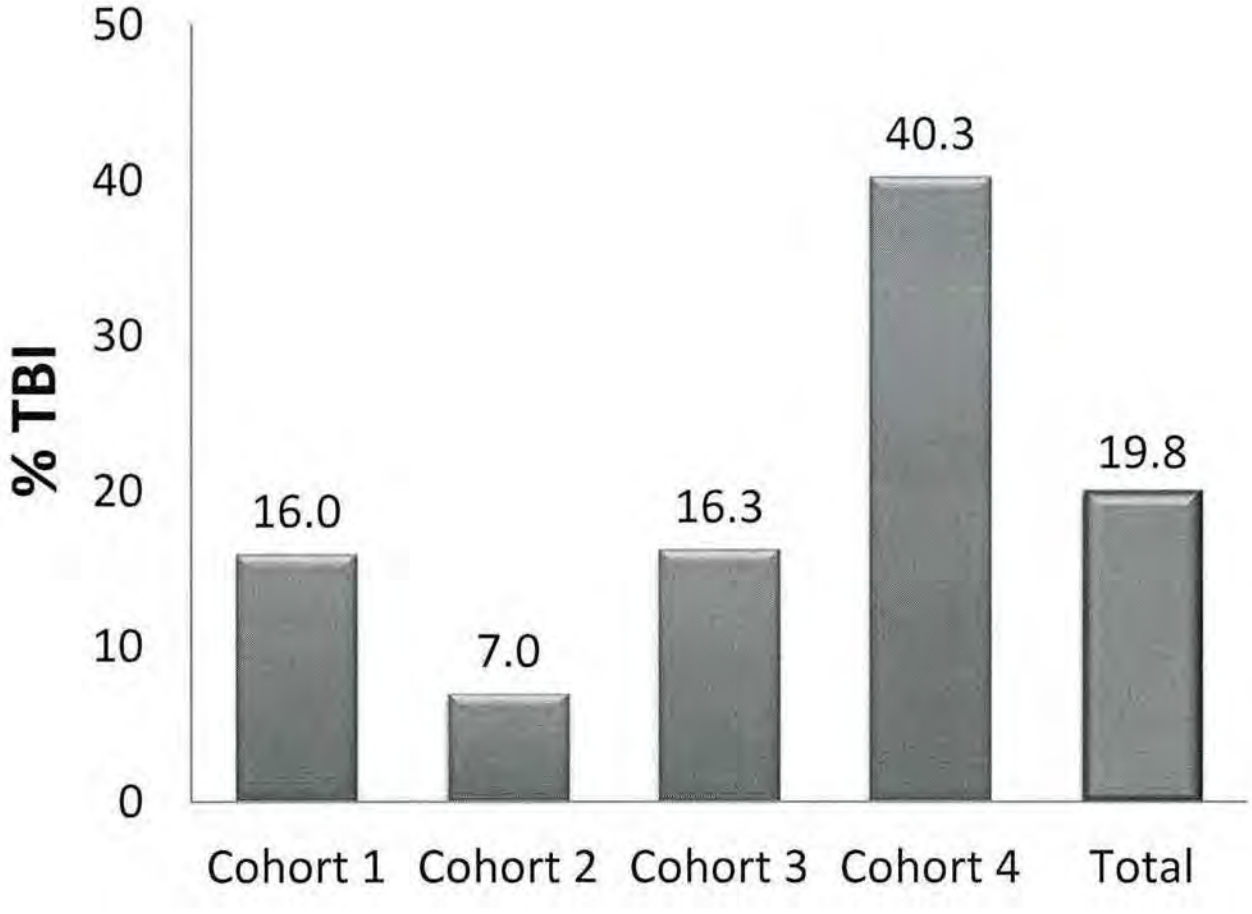
Combat Experiences Scale



Vogt et al (2008) For Comparison

- N=640 Army soldiers, Iraq 2003-2004
- Combat/combat support
- Combat Experience Score Mean(SD) = **34.7(10.4)**

Deployment-related TBI endorsement was variable across deployments, but was high in some battalions



Combat Experience Scale	
Cohort 1	6.5
Cohort 2	6.9
Cohort 3	13.2
Cohort 4	22.7
Total	**12.9**

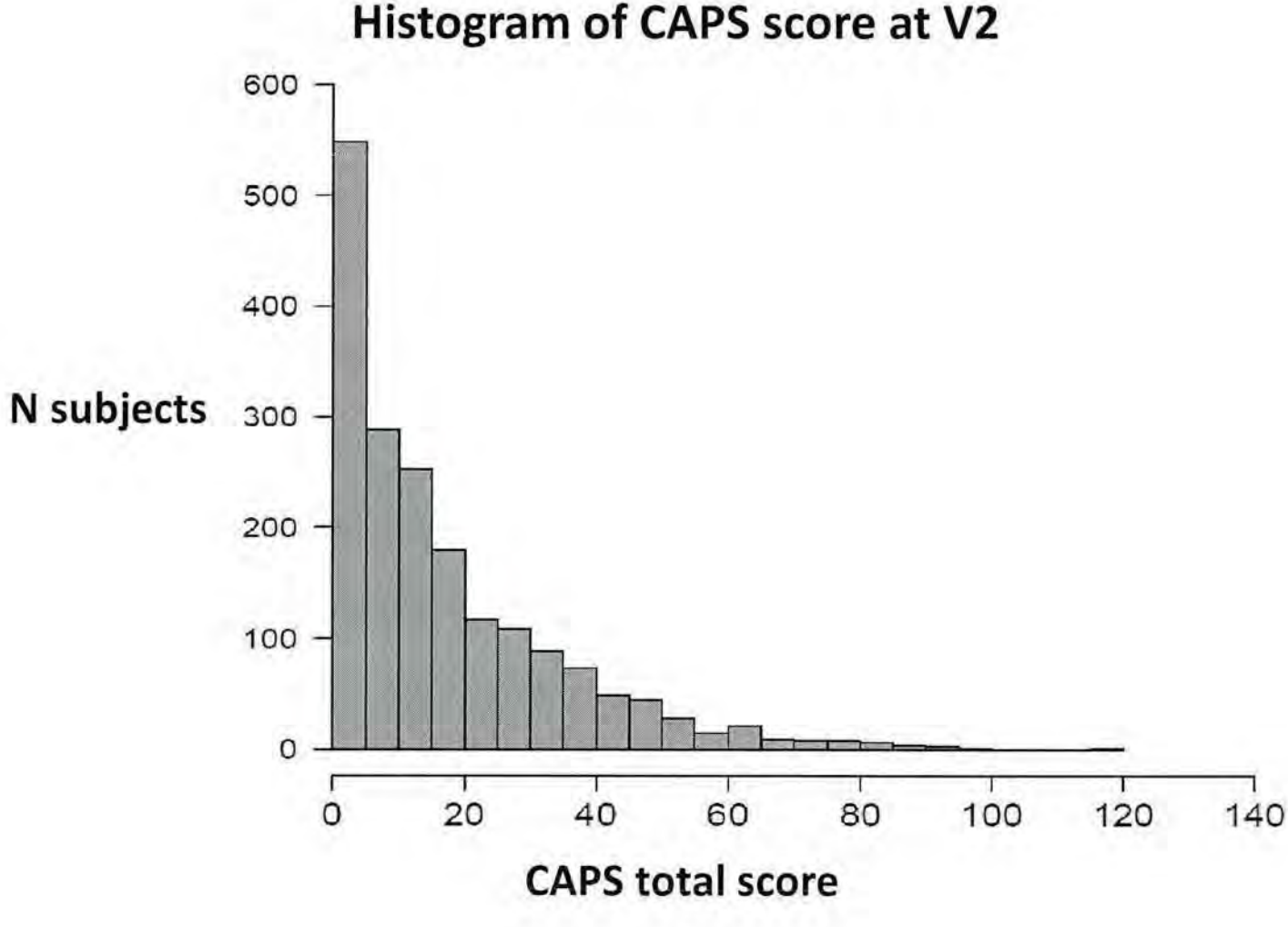
Clinician Administered PTSD Scale (CAPS)

- The MRS uses the CAPS as the primary measure of PTSD symptoms and total symptom burden.
- The CAPS is a structured interview designed to provide both continuous and dichotomous data about symptoms.

4 Symptom Clusters	CAPS Scale Range
Re-experiencing	0-40
Avoidance	0-32
Emotional Numbing	0-24
Hyperarousal	0-40
Total score	0-136

ZINB Distribution

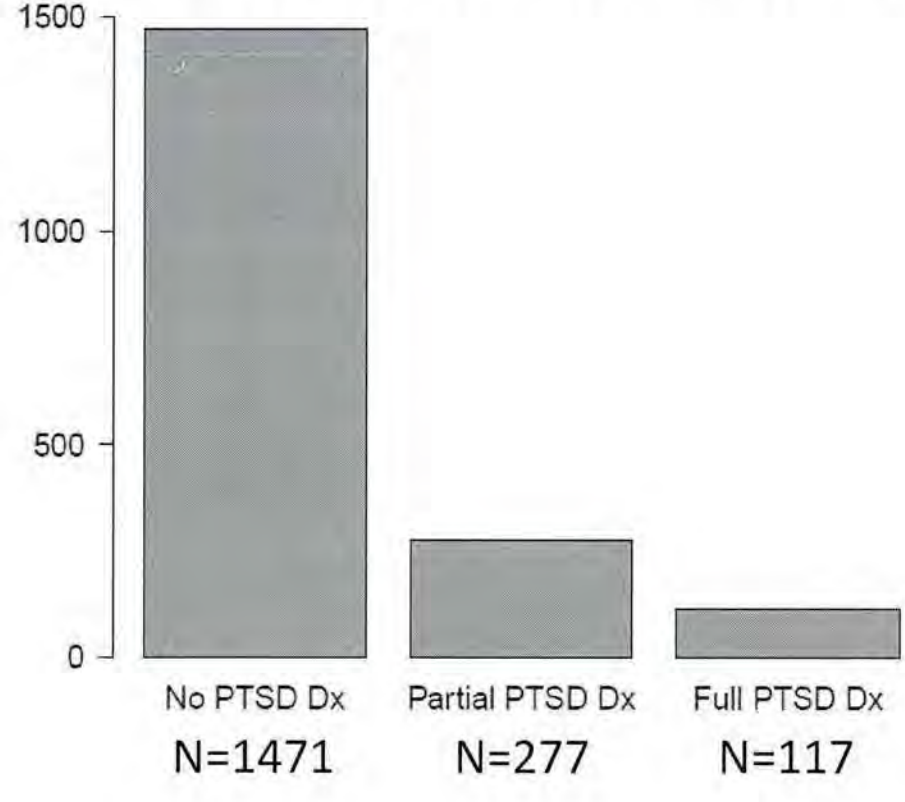
- CAPS total score is not a normally distributed trait in MRS. The trait cannot simply be transformed to normality because there are too many zero value scores
- Zero inflated negative binomial (ZINB) distribution: ZINBR best statistical model



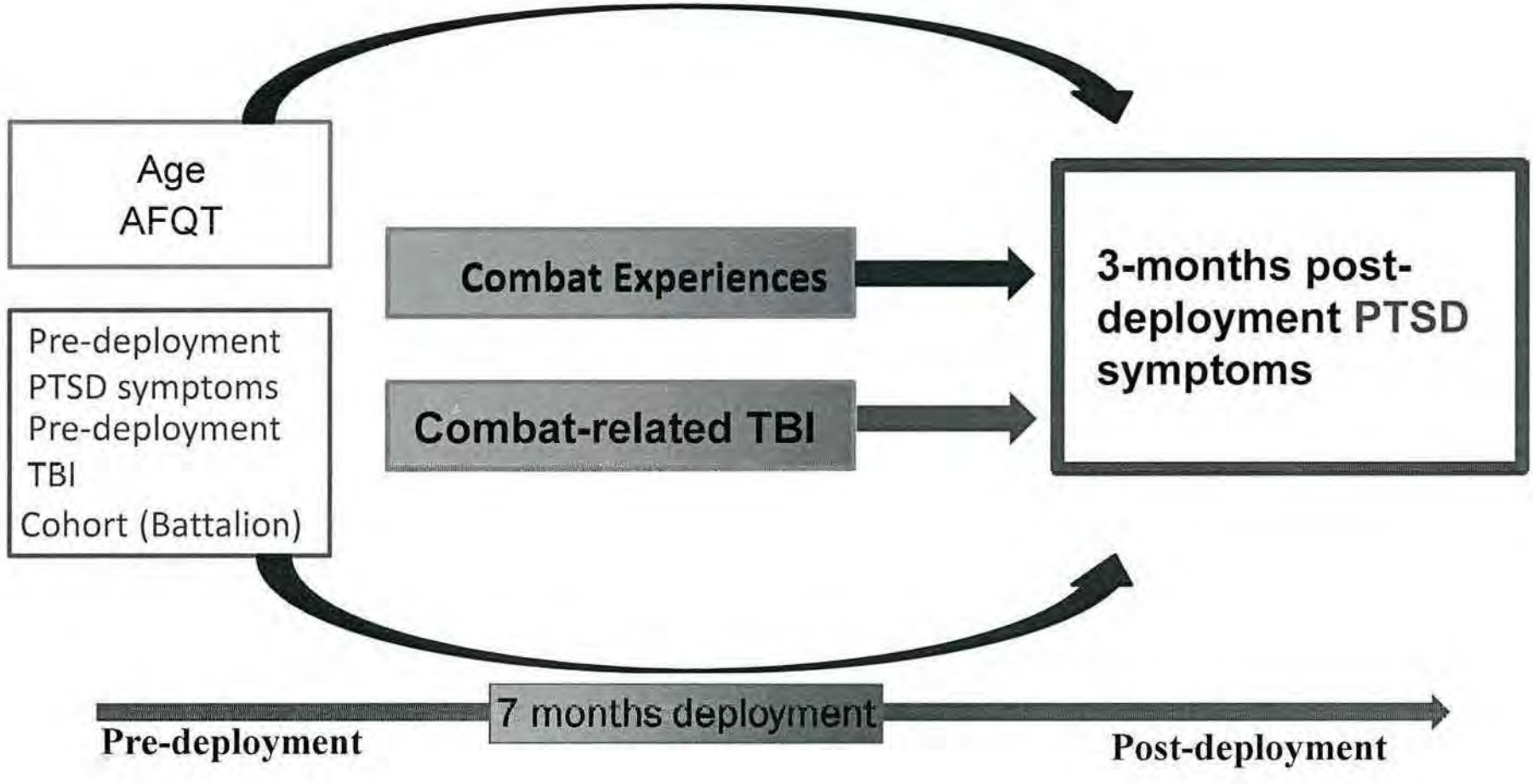
Grouping subjects using CAPS DSM-IV diagnosis:

- Alternatives to modeling based upon raw score (CAPS total), we can use diagnosis to group subjects and use ordered logistic regression to model the data
- There are 3 groups in order of severity: No diagnosis, partial PTSD diagnosis (stringent or lenient criteria), or the DSM-IV based PTSD diagnosis

Quantity of subjects within each CAPS group at V2



Prospective analysis of deployment-related combat stress and TBI on Post-deployment PTSD



Original Investigation

Association Between Traumatic Brain Injury and Risk of Posttraumatic Stress Disorder in Active-Duty Marines

Kate A. Yurgil, PhD; Donald A. Barkauskas, PhD; Jennifer J. Vasterling, PhD; Caroline M. Nievergelt, PhD; Gerald E. Larson, PhD; Nicholas J. Schork, PhD; Brett T. Litz, PhD; William P. Nash, MD; Dewleen G. Baker, MD; for the Marine Resiliency Study Team

JAMA Psychiatry. 2014;71(2):149-157. doi:10.1001/jamapsychiatry.2013.3080
Published online December 11, 2013.

J Head Trauma Rehabil

Prospective Associations Between Traumatic Brain Injury and Postdeployment Tinnitus in Active-Duty Marines

Kate A. Yurgil, PhD; Royce E. Clifford, MD, MPH; Victoria B. Risbrough, PhD; Mark A. Geyer, PhD; Mingxiong Huang, PhD; Donald A. Barkauskas, PhD; Jennifer J. Vasterling, PhD; MRS Team; Dewleen G. Baker, MD

J Head Trauma Rehabil. 2015 Feb 19.
PubMed PMID: 25699623.

MRS Hemodynamics

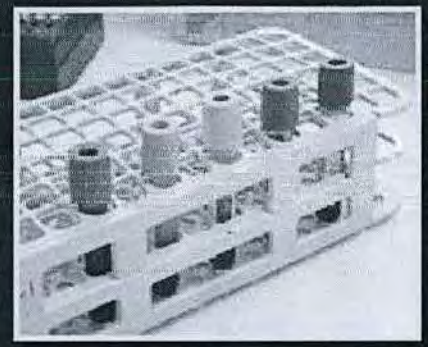
Cardio-Metabolic function before and after combat stress

Daniel T. O'Connor

Dynapulse station

MRS Biobank

Samples collected and processed on site:



- Urine: 6x 2ml cryovials with almost 2ml urine
- Saliva: 4x 2ml cryovials with at least 1ml saliva
- Blood:

  Plasma samples:

  2x 4ml Lithium Heparin tubes (green tops); 4 aliquots

  1x 6ml EDTA tubes (purple tops); 2 aliquots

  Plasma samples are centrifuged at 4°C, 3000rpm for 15 min; keep supernatant

  RNA samples:

  1x 10ml EDTA tubes (purple tops) for RNA processing (Ming Tsuang –lab)

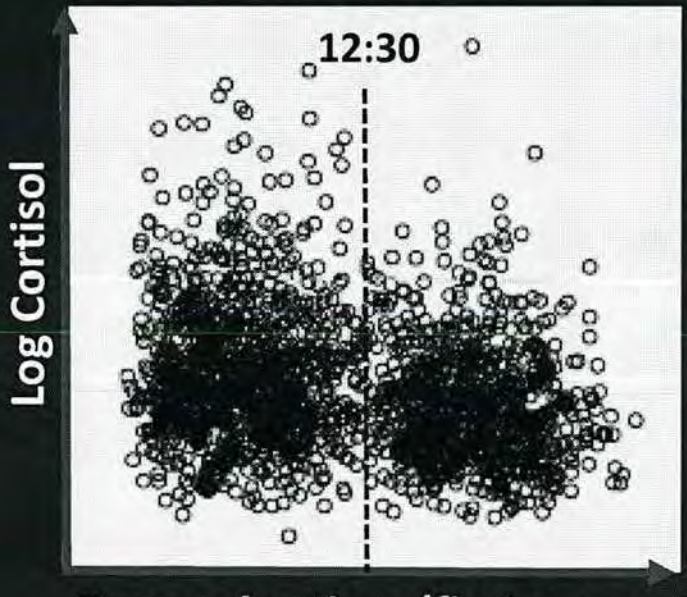
  DNA whole-blood samples:

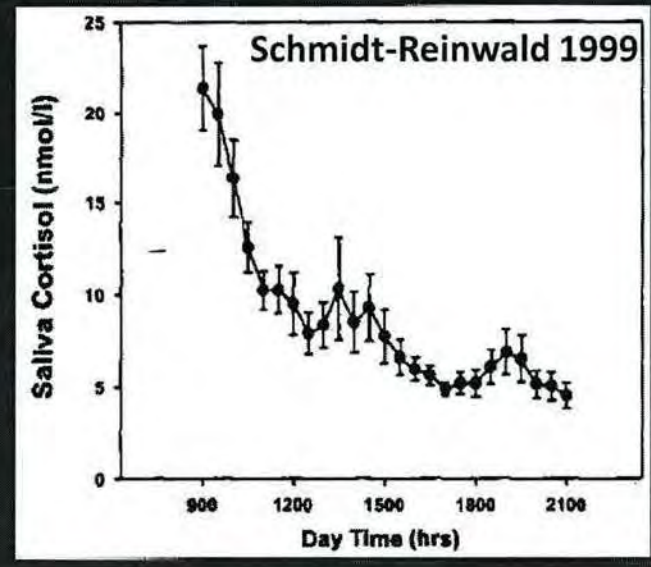
  1x 10ml EDTA tubes (purple tops) for whole blood DNA (6/4 cryovials with ~1ml blood)

Samples are kept on dry ice during processing and stored in freezer at -80° C

MRS Sample collection

Salivary cortisol: Circadian Rhythm





ANOVA: dependent = logCortisol
N= 1695
R= -0.25; p<0.00001
Age-adjusted

No significant correlation with dynapulse time was found for:

- log Salivary Cotinine: p>0.68
- log Plasma CRP: p>0.71
- log Salivary Alpha-amylase: p>0.36

JAMA Psychiatry

Research

Original Investigation

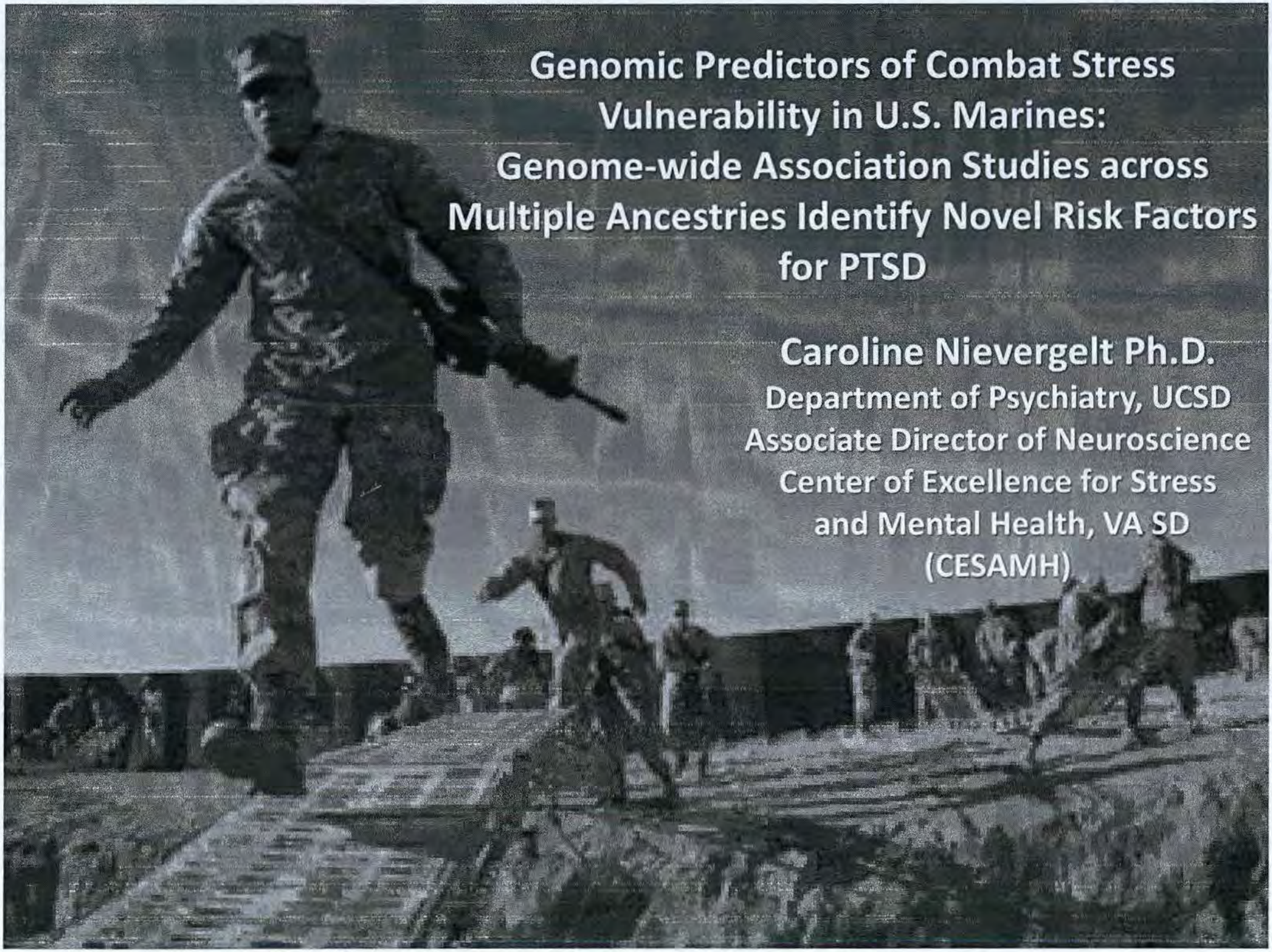
Assessment of Plasma C-Reactive Protein as a Biomarker of Posttraumatic Stress Disorder Risk

Satish A. Eraly, MD, PhD; Caroline M. Nievergelt, PhD; Adam X. Maihofer, MS; Donald A. Barkauskas, PhD; Nilima Biswas, PhD; Agorastos Agorastos, MD; Daniel T. O'Connor, MD; Dewleen G. Baker, MD; for the Marine Resiliency Study Team

MAIN OUTCOMES AND MEASURES Severity of PTSD symptoms 3 months after deployment assessed by the Clinician-Administered PTSD Scale (CAPS).

CONCLUSIONS AND RELEVANCE A marker of peripheral inflammation, plasma CRP may be prospectively associated with PTSD symptom emergence, suggesting that inflammation may predispose to PTSD.

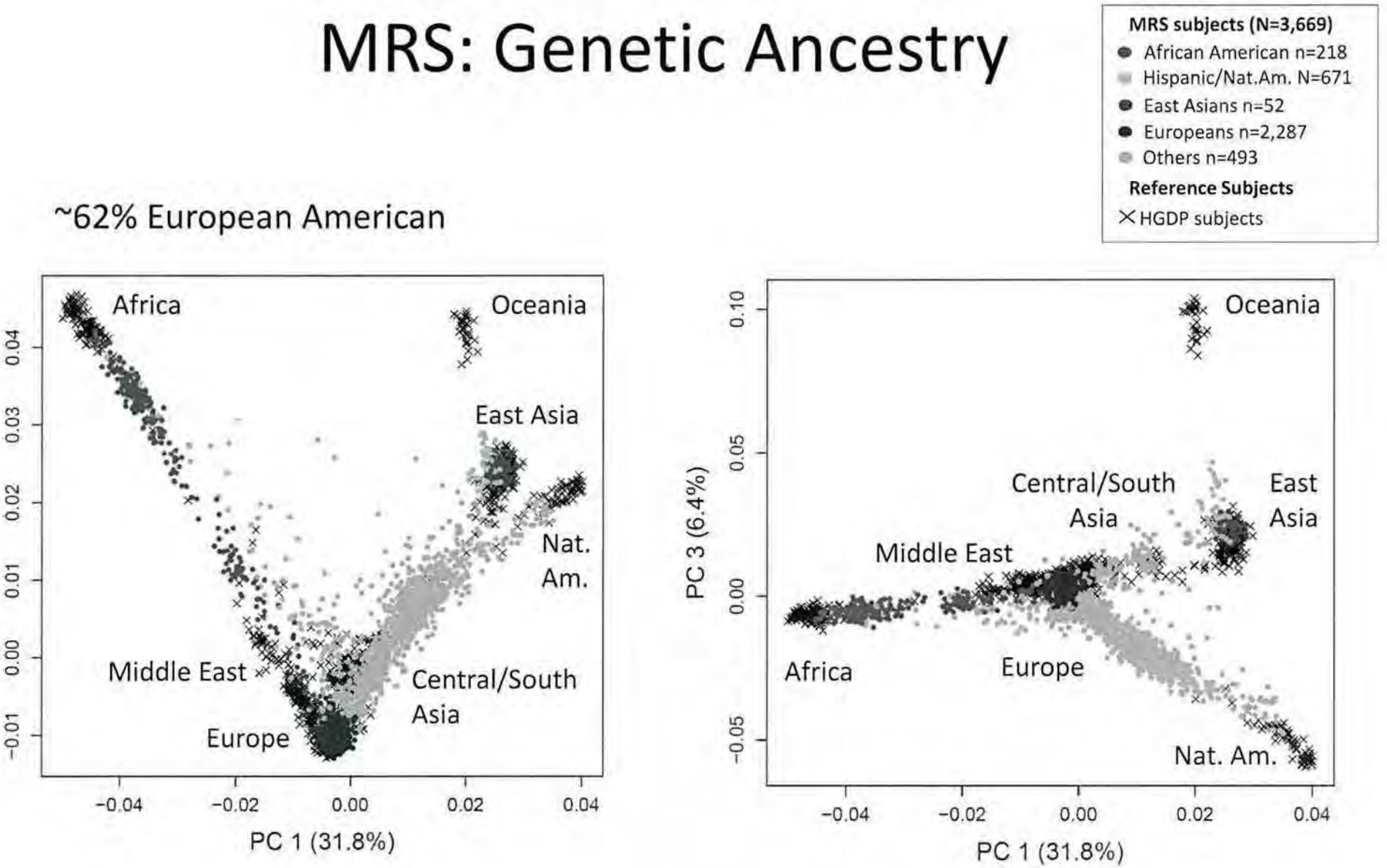
JAMA Psychiatry. 2014;71(4):423-431. doi:10.1001/jamapsychiatry.2013.4374
Published online February 26, 2014.



Genomic Predictors of Combat Stress Vulnerability in U.S. Marines: Genome-wide Association Studies across Multiple Ancestries Identify Novel Risk Factors for PTSD

Caroline Nievergelt Ph.D.
Department of Psychiatry, UCSD
Associate Director of Neuroscience
Center of Excellence for Stress
and Mental Health, VA SD
(CESAMH)

MRS: Genetic Ancestry



- Bayesian based cluster methods (STRUCTURE) to generate ancestry estimates based on HGDP reference populations and AIMs
- Determination of main ancestral groups (<5% admixture)
- Visual inspection: PCA with reference populations and color coding for main ancestral groups

Psychoneuroendocrinology (2015) 51, 459–471

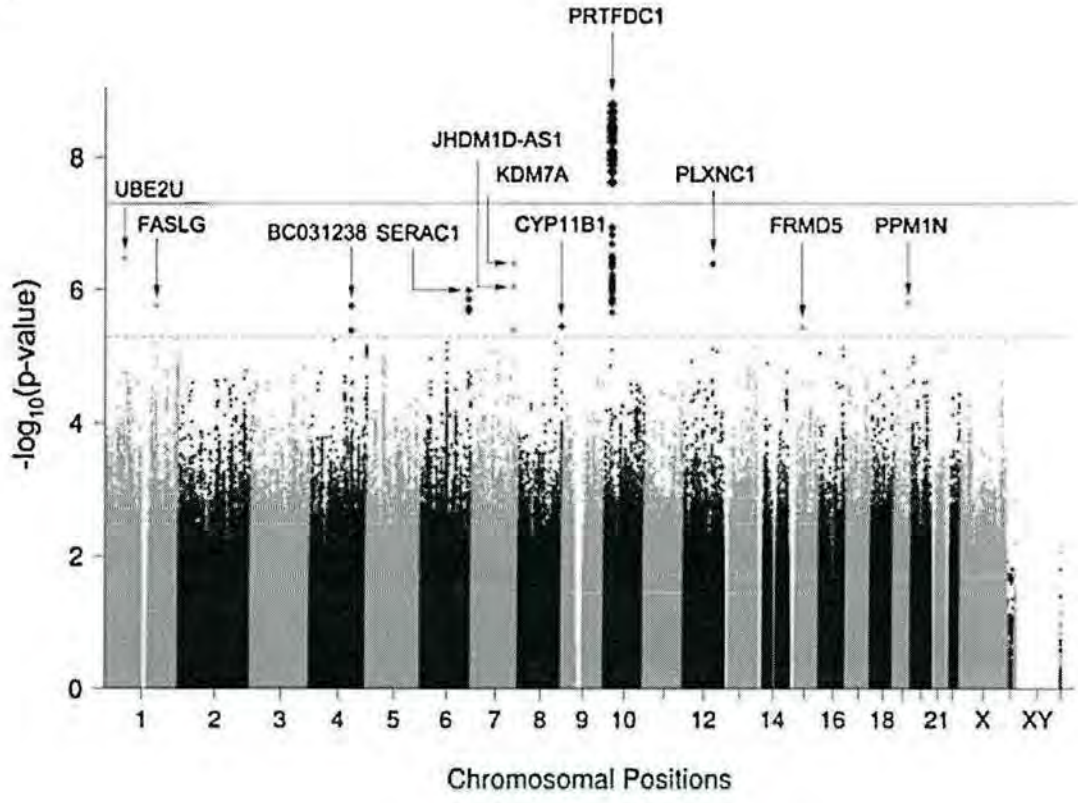
Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/psyneuen

Genomic predictors of combat stress vulnerability and resilience in U.S. Marines: A genome-wide association study across multiple ancestries implicates *PRTFDC1* as a potential PTSD gene

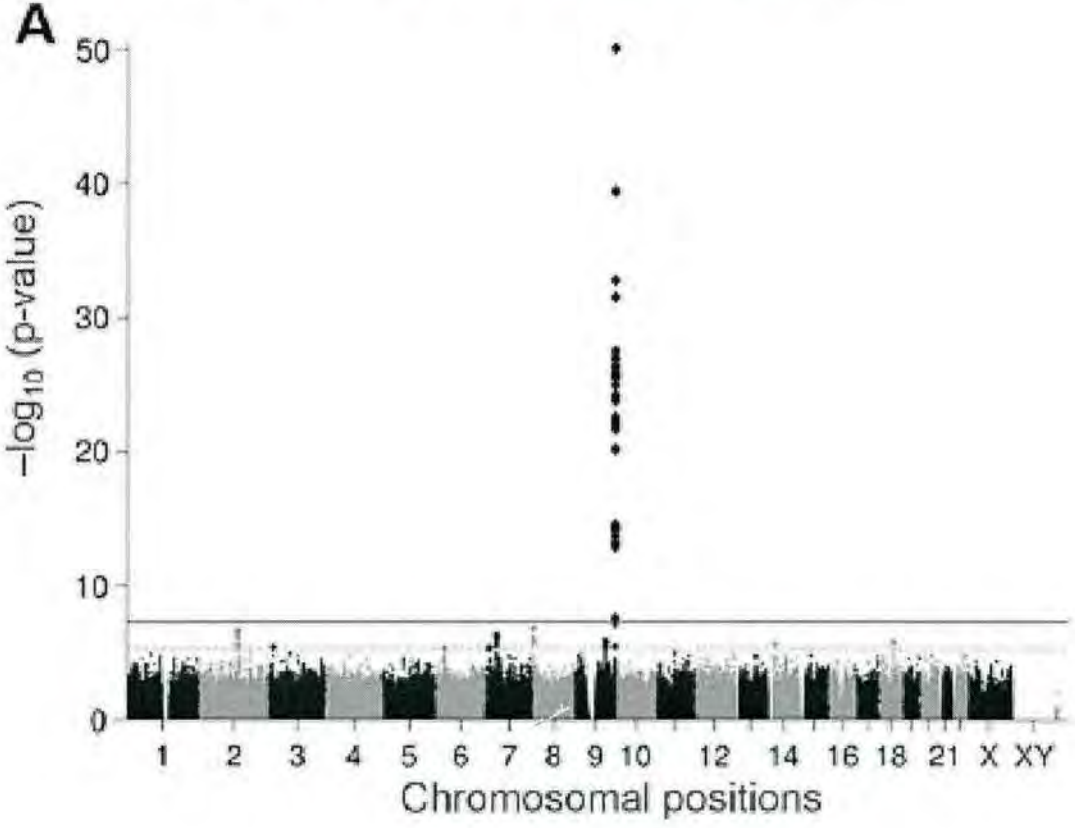
Caroline M. Nievergelt[a,d,*], Adam X. Maihofer[a], Maja Mustapic[a,b], Kate A. Yurgil[d], Nicholas J. Schork[c], Mark W. Miller[e,f], Mark W. Logue[g,h], Mark A. Geyer[a], Victoria B. Risbrough[a,d], Daniel T. O'Connor[b], Dewleen G. Baker[d,a]



Human Molecular Genetics

The catecholamine biosynthetic enzyme dopamine β-hydroxylase (DBH): first genome-wide search positions trait-determining variants acting additively in the proximal promoter

Maja Mustapic[1,2,4], Adam X. Maihofer[1], Manjula Mahata[2], Yuqing Chen[2], Dewleen G. Baker[1,3], Daniel T. O'Connor[2] and Caroline M. Nievergelt[1,3,*]



Hum. Mol. Genet. (2014) 23 (23): 6375-6384

MRS Genomic and Data Integration

- DNA and RNA isolated from peripheral blood leukocytes
- 60 (future) PTSD cases and 60 trauma-exposed controls
- Timepoints: at pre-deployment, and 3- and 6-months post-deployment
- Epigenome: Genome-wide methylation (Illumina 450K)
- Transcriptome: Genome-wide gene expression (RNAseq)
- Transcriptome 2: RNA array data on additional MRS subjects

RAPID PUBLICATION

AMERICAN JOURNAL OF medical genetics
Neuropsychiatric Genetics

Blood-Based Gene-Expression Predictors of PTSD Risk and Resilience Among Deployed Marines: A Pilot Study

Stephen J. Glatt,[1*] Daniel S. Tylee,[1] Sharon D. Chandler,[2] Joel Pazol,[2] Caroline M. Nievergelt,[3,4,5] Christopher H. Woelk,[4,6] Dewleen G. Baker,[3,4,5] James B. Lohr,[3,4,5] William S. Kremen,[2,3,5] Brett T. Litz,[7] Ming T. Tsuang,[2,3,4,5,8,9,10] and Marine Resiliency Study Investigators

Molecular Psychiatry (2015), 1–8

www.nature.com/mp

ORIGINAL ARTICLE

Gene networks specific for innate immunity define post-traumatic stress disorder

MS Breen[1], AX Maihofer[2], SJ Glatt[3], DS Tylee[3], SD Chandler[2], MT Tsuang[2,4,5,6,7], VB Risbrough[2,4], DG Baker[2,4], DT O'Connor[5,8], CM Nievergelt[2,4,9] and CH Woelk[1,9]

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Psychiatric and medical
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- **GWAS** (complete data)
- **Methylome** (subset, pre-post)
- **Transcriptome** (subset, pre-post)

***GWAS UCSD,NIH R0-1 Caroline Nievergelt PI**
***Gene expression, UCSD, R21 Ming Tsuang PI**
***Methylome, RNA-seq C Nievergelt MRS-II**

Psychophysiology and Neurocognitive Projects

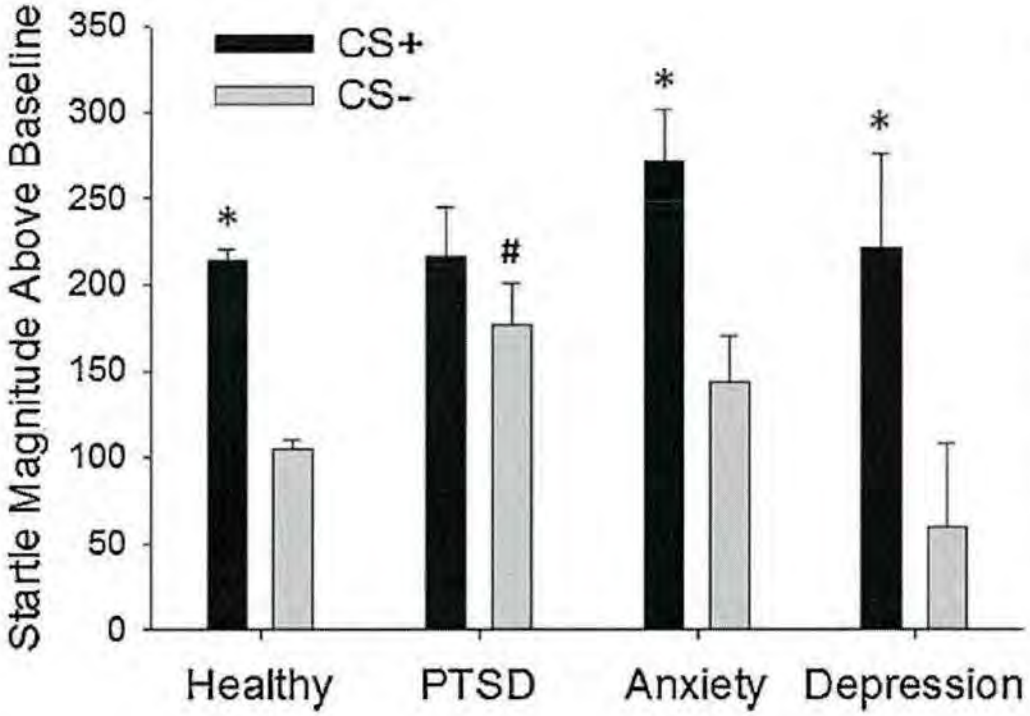
Directors: Vickie Risbrough and Mark Geyer

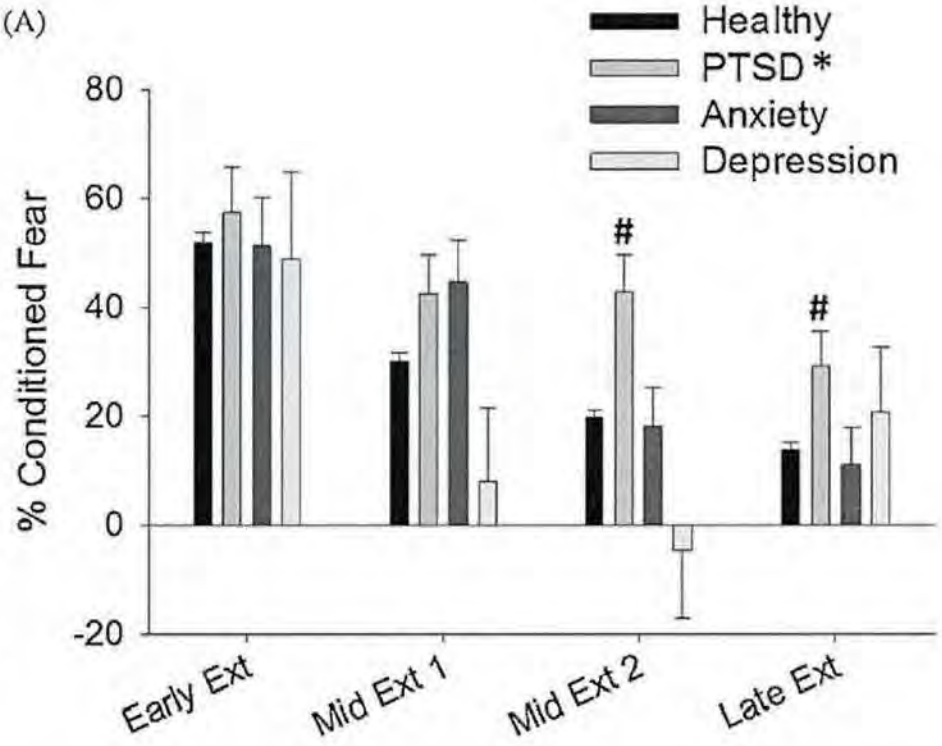
Construct/Task	Collaborators
Fear Conditioning and Extinction/FPS	Dean Acheson
Startle Threshold/EMG	Dan Glenn
Prepulse Inhibition/EMG	Dean Acheson
Heart Rate Variability/PPG	Arpi Minassian
Reaction time/ANAM+Penn Battery	Gur, Moore, Vasterling
Attention/CPT, Go-NoGo	Ruben Gur, Tyler Moore
Attention Set Shifting	Ruben Gur, Tyler Moore
Verbal, Spatial, Facial Memory	Ruben Gur, Tyler Moore
Spatial and Verbal Reasoning	Ruben Gur, Tyler Moore
Working Memory	Ruben Gur, Tyler Moore

Example Aims

- Identify psychophysiological predictors of PTSD
- Identify deployment-related TBI effects on changes in neurocognition
- Identify potential mechanism of TBI-induced increases in risk for PTSD
- Identify overlapping genetic mechanisms of PTSD risk and endophenotypes (fear conditioning, extinction and prepulse inhibition, HRV)

Poor safety signal learning and extinction are biobehavioral markers of PTSD





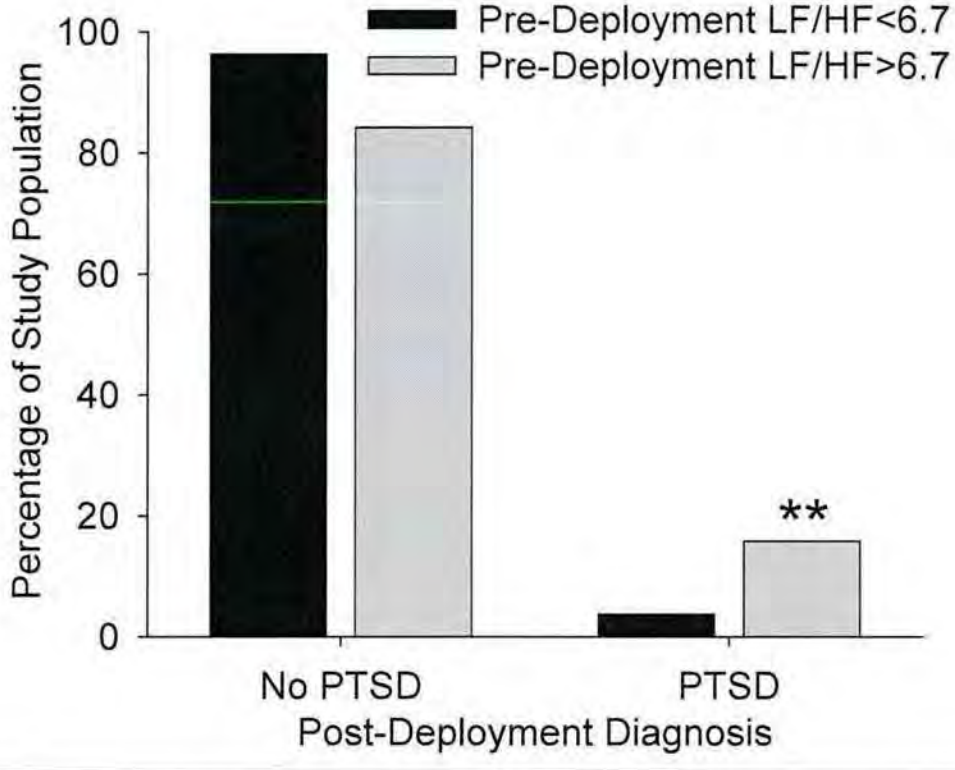
Conditioned fear and extinction learning performance and its association with psychiatric symptoms in active duty Marines.

Acheson DT, Geyer MA, Baker DG, Nievergelt C, Yurgil K, Risbrough VB (2015)

Psychoneuroendocrinology 51:495-505

Pre-trauma low HRV is associated with PTSD risk

Parameter	OR	95% CI	P
Trauma exposure	2.84	2.15-3.74	<0.001
LF: HF ratio	1.47	1.10-1.98	0.01



Association of predeployment heart rate variability with risk of postdeployment Postraumatic Stress Disorder in active-duty Marines

Arpi Minassian, Adam Maihofer, Dewleen Baker, Caroline Nievergelt, Mark Geyer, and Victoria Risbrough. JAMA Psychiatry 2015;72(10):979-98

Actionable Results

Psychological , Behavioral and Physiological assessments

Mental and Physical Health
- Deployment related TBI predictors PTSD – Follow-up data collection

Psychophysiology and Neurocognition
- Pre-deployment HRV predicts PTSD
- Sensorimotor gating and cue discrimination may be resiliency factors for PTSD

MRS
Secure database

Career History Archival Medical and Personnel System database

VA and military service database information in follow-up data collection and analysis

Biobank

Stored samples for future analyses and collaborations

Biological assessments

Biomarkers
- Pre-deployment immune activation state predicts PTSD – replicate findings, integrate analysis with HRV

Imaging
- Development of diagnostic MEG signatures for mTBI

Genomics
- PGC PTSD GWAS consortium
- PGC PTSD EWAS consortium
- Active replication of GWAS and EWAS study findings in collaboration with PRISMO, Army STARRS, Grady Trauma Cohort

Ongoing and Future Directions

- **Integrated data analyses of MRS data sources,** e.g. behavior, genomics, metabolomics, physiology, imaging

- **Future data collection: MRS-III 5-year follow up**
- **Funded:** CDMRP (DoD), FY 2016-2019, PI: Baker, TBI/Tinnitus Study
  Long term outcomes –hearing, tinnitus, health
  Better understand HPA axis/autonomic – vagal/immune system relationships in PTSD – TLR-4, TLR-9, $\alpha 7$ nicotinic receptor

- **TBI/PTSD Imaging Biomarker Validation and Development**
- **Funded:** Investigating the Neurologic Effects of Training Associated Blast (I-TAB) study
  Longitudinal imaging of blast-exposed trainees

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

Form Approved
OMB No. 9000-0095
Expires Jan 31, 2008

PLEASE DO NOT RETURN YOUR COMPLETED FORM TO THE ABOVE ORGANIZATION. RETURN COMPLETED FORM TO THE CONTRACTING OFFICER.

1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
Veterans Medical Research Foundation	N62645-11-C-4037	Same	Same	a. INTERIM [X] b. FINAL
b. ADDRESS (Include ZIP Code)	**d. AWARD DATE** (YYYYMMDD)	**b. ADDRESS** (Include ZIP Code)	**d. AWARD DATE** (YYYYMMDD)	**4. REPORTING PERIOD** (YYYYMMDD)
3350 La Jolla Village Drive, MC 151A San Diego, CA 92161	20110916	Same		a. FROM 20110916 b. TO 20160331

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state)

NAME(S) OF INVENTOR(S) (Last, First, Middle Initial) a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS (X) d. (1) UNITED STATES (a) YES	(1) UNITED STATES (b) NO	(2) FOREIGN (a) YES	(2) FOREIGN (b) NO	CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (X) e. (a) YES	(b) NO
Robert Naviaux/Dewleen Baker	Diagnostic and Predictive Metabolite Patterns for Disorders Affecting the Brain and Nervous System	SD2014-043	X				X	

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(2) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER (Include ZIP Code)	(c) ADDRESS OF EMPLOYER (Include ZIP Code)		

SECTION II - SUBCONTRACTS (Containing a "Patent Rights" clause)

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR (If "None," so state)

NAME OF SUBCONTRACTOR(S) a.	ADDRESS (Include ZIP Code) b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d. (1) CLAUSE NUMBER	(2) DATE (YYYYMM)	DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES (YYYYMMDD) f. (1) AWARD	(2) ESTIMATED COMPLETION
Trustees of the University of Pennsylvania	3451 Walnut Street, P-221, Philadelphia, PA 19104-6205	28345-D1	52.227.11	201210	Identify, assemble the computerized battery to be used. Data analysis/interpret	20121001	20131231
Boston VA Research Institute, Inc.	150 S. Huntington Avenue, Boston, MA 02130-4817	28345-S03	52.227.11	201008	Preparation work before data handling. Initial data analysis/Cross sectional data.	20120801	20130930

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR (Not required if: (X as appropriate))

SMALL BUSINESS or [X] NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, Middle Initial)	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Munoz Canete, Alma	Contracts and Grants Director	Alma Munoz Canete	7/7/2016