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Levels of pituitary adenylate cyclase-activating polypeptide (PACAP) in posttraumatic stress disorder (PTSD) and the modulatory effect of vagus nerve stimulation (VNS)

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ABSTRACT

Levels of pituitary adenylate cyclase-activating polypeptide (PACAP) in post-traumatic stress disorder (PTSD) and the modulatory effect of vagus nerve stimulation (VNS)

By Yunshen Jiao

Background
Post-traumatic stress disorder (PTSD) is a mental disorder with 6.8% prevalence in the U.S. Evidence has shown that PACAP may be disturbed in PTSD. There is a lack of studies in humans examining longitudinal changes in PACAP in relation to experimentally-controlled trauma recall or other stressful paradigms. Vagus nerve stimulation is used experimentally in treating neuropsychiatric disorders. However, no study to date has examined whether VNS would improve the quality of life for patients with PTSD, or whether the up-regulation of PACAP found with PTSD can be decreased or modulated by VNS in humans.

Methods
This study is part of the project *Closed Loop Vagal Stimulation in Patients with Posttraumatic Stress Disorder (Dr. JD Bremner, PI, BD Pearce, Co-I, Y Ko, Co-I)*. A total of 12 patients with PTSD and 24 healthy subjects had a baseline psychological and health assessment. Then, a procedure which elicits trauma-related stress responses was conducted. Patients were randomly assigned to VNS treatment or sham stimulation and blood was collected at baseline and during the procedure. Linear regression and mixed models were used to assess the association between PACAP and other biomarker blood levels, psychological tests scores, PTSD diagnosis, and VNS treatment.

Results
Adjusted for age, sex, BMI, race, and education level, PACAP blood levels continuously increased during the procedure (1st day: 9%, 95%CI: (1.00, 1.18); 2nd day: 14%, 95%CI: (1.04, 1.25); 3rd day: 15%, 95%CI: (1.03, 1.28)). This increase was consistently lower in the VNS compared to the sham stimulated group. The log-transformed PACAP concentration was significantly, positively associated with the log-transformed concentration of IFN-γ (β=0.14, p=0.022) and Ghrelin (β=0.15, p=0.05), and significantly negatively correlated with IL6 (β=-0.13, p=0.048), IL13 (β=-0.25, p=0.006), and IL5 (β=-0.27, p=0.013).

Conclusions
This is the first report of PACAP in humans undergoing a trauma recalls paradigm. Trauma or stressful tasks were associated with increased PACAP blood levels. However, the association in VNS treatment to PACAP blood levels still needs to be established in a Phase 2 study. Combined with correlated inflammatory cytokines, PACAP may be a biomarker to show or predict the treatment effect of VNS to PTSD.

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ABBREVIATIONS

Abbreviation	Explanation
BMI	Body Mass Index
BNST	Bed nucleus of the stria terminalis
BUN	Blood urea nitrogen
CBC	Complete blood count
DARPA	Defense Advanced Research Projects Agency
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th Edition, Text Revision
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision
EEG	Electroencephalography
EKG	Electrocardiography
HPA	Hypothalamic-pituitary-adrenal
IFN-γ	Interferon γ
IL	Interleukin
nVNS	noninvasive Vagus nerve stimulation
PACAP	Pituitary adenylate cyclase-activating polypeptide
PET	Positron emission tomography
PTSD	Posttraumatic stress disorder
SCID	Structured Clinical Interview for the DSM
VNS	Vagus nerve stimulation
In Formula	
$PACAP$	Blood levels of PACAP
$PTSD$	PTSD diagnosis status. Meet criteria for PTSD coded as "1", otherwise as "0"
Sex	Biological sex of the subject. Females coded as "1", males coded as "0"
$Race$	The race of the subject. White / Caucasian coded as "0", African American coded as "1", other races coded as "2"
$Education$	Education level of the subject. Associate's degree or lower coded as "1", Bachelor's degree or higher coded as "0"
$Scores$	The score of a specified psychological scale
$Dich\_Scores$	Psychometric scores dichotomized at the median with the lower half coded as "0" and upper as "1'
$Treat$	Receiving sham stimulation coded as "0", receiving VNS treatment coded as "1"
$Time1$	Blood draw taken during day 1 procedure coded as "1". Blood draw at baseline or in other days coded as "0"
$Time2$	Blood draw taken during day 2 procedure coded as "1". Blood draw at baseline or in other days coded as "0"
$Time3$	Blood draw taken during day 3 procedure coded as "1". Blood draw at baseline or in other days coded as "0"
$Biomarker$	The blood levels of a specified biomarker

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INTRODUCTION

Post-Traumatic Stress Disorder (PTSD) is a mental disorder which occurs in some people who have encountered or witnessed a terrifying event (1). The National Comorbidity Survey Replication (NCS-R) estimated the lifetime prevalence of PTSD among adult Americans to be 6.8% using DSM-IV criteria; and women (10.4%) were twice as likely as men to experience PTSD (5%) during their lifetime (2). In addition to substantial loss of human capital due to disability or decreased productivity caused by PTSD, the economic burden of PTSD is also tremendous, resulting in about an average loss of $2.0 to $3.1 billion per year in the U.S.(3). Despite the tremendous burden of PTSD, the treatment is still not widespread or effective (4). According to the World Mental Health Surveys (WMH), only half of people with PTSD had sought any kind of treatment in high-income countries, and the proportion is much smaller in the middle- or low-income countries (5). Generally, the first-line treatment for PTSD is psychotherapy, especially trauma-focused and non-trauma-focused cognitive behavior therapy (CBT) (6). While traditional treatments are available in the U.S., about ⅓ of patients with PTSD still have symptoms associated with PTSD (7). In addition, 40% to half of the patients do not respond or only partially respond to anti-depressant medication (6, 8). Moreover, regardless of which treatment method is chosen, about 40% of the subjects have a recurrence of symptoms within one year, and the risk of five-year recurrence is about 20% (9). These findings indicate patients either do not receive treatment or that traditional treatments are not effective over a prolonged period of time. Therefore, more research is focused on exploring the neuropathological mechanisms of PTSD and developing new targeted treatments.

Neuroendocrine and immune connections to PTSD

Both acute and chronic stress exert influence on the hypothalamic-pituitary-adrenal (HPA) axis, and the corticotrophin releasing factor (CRF) is the key stress neurotransmitter for stimulation of the central sympathetic and serotonergic systems (10). CRF receptors are wildly expressed on the limbic regions of the brain, hypothalamus and immune cells. The key factors involved in the regulation of homeostasis in the HPA axis are glucocorticoids, neurotransmitters, norepinephrine and serotonin, and pro-inflammatory cytokines (10).

It is known that the HPA axis plays an important role in the pathophysiology of stress-related psychiatric disorders, while the HPA axis and the immune system are interrelated (11). Passos and colleagues (12) have conducted a meta-analysis of published studies showing that there is an increased level of inflammatory markers, including interleukin 6 (IL6), interleukin 1β (IL1β), TNFα, and interferon γ (IFN-γ), in patients with PTSD compared to the healthy controls. In addition, Ressler *et al* (13) found that high levels of pituitary adenylate cyclase activating polypeptide (PACAP), a molecule that regulates cellular, neural and cardiovascular stress responses, is associated with female PTSD. Likewise, the PACAP/PAC1 receptor expression and signaling may participate in the psychological and physiological responses to regulate traumatic stress. Moreover, a growing literature on the anatomical location and physiology of PACAP suggests a close association with systems that are also regulated by VNS. However this has not been previously examined and is the subject of this thesis.

Starr and Margiotta provided an excellent review of the cellular and molecular physiology of PACAP. A variety of *in vitro* and *in vivo* experiments demonstrated that PACAP is extensively connected to physiological stress responses including those mediated by the sympathetic and parasympathetic nervous system. Available data suggested that

PACAP is released along with acetylcholine from nerve terminals in the adrenal gland, and targets cells that express the nicotinic receptor along with PACAP receptors. Accordingly, there was evidence for a functional adrenomedullary synapse in which acetylcholine and PACAP act independently to cause release of catecholamines (14).

Within the brain, PACAP participates in neural circuits relevant to PTSD and other stress disorders. In the hypothalamus and hippocampus, PACAP was an important neuro-modulator, though many of the details are still under investigation (15, 16). Numerous anatomical and physiological studies had established the importance of PACAP neurotransmission in the amygdala, which underscored the role of this peptide transmitter in PTSD. Of particular interest to VNS, PACAP innervation of the lateral central amygdala is thought to arise from PACAP containing neurons in the vagus nerve brainstem complex. Behavioral studies in rodents indicate that PACAP exerts an anxiogenic effect via its connections in the BNST (17). Therefore, PACAP is exceptionally well positioned as a mediator in pathways of the cholinergic anti-inflammatory axis and its potential modulation by VNS.

VNS in the treatment of mental illness

Based on the fact that PTSD is a disorder comprising molecular and neurochemical changes in the brain, (18), a series of neuromodulator-based treatments have been proposed and are in various stages of development and application. These include: repetitive transcranial magnetic stimulation (rTMS), transcranial direct current stimulation (tDCS), trigeminal nerve stimulation (TNS), deep brain stimulation (DBS), and vagus nerve stimulation (VNS). This thesis focuses on VNS, which has been experimentally applied to the treatment of various mental illnesses (19).

In 1997, the first device panel of VNS was approved by the US FDA for the treatment of epilepsy. During the following decades, VNS has been used to treat drug-resistant epilepsy and treatment-resistant depression (20), and a growing body of research has been devoted to the exploration of VNS for the treatment of other refractory mental disorders. Experiments in rats (21) suggested that VNS may be an effective adjunct to exposure therapy for the treatment of PTSD.

Considering the serious side effects of invasive VNS, including the early side effects associated with the implantation surgery (hoarseness, difficulty in breathing and swallowing) and late complications related to the device and to stimulation of the vagus nerve (arrhythmia, laryngeal dysfunction, obstructive sleep apnea, etc.) (22), new medical devices were developed with selective and non-invasive stimulation to the vagus nerve fibers. There are two categories of non-invasive VNS devices: transcutaneous electrical stimulation of the cutaneous receptive field of the auricular branch of the vagus nerve in the outer ear (NEMOS®, NET-3000, etc.), and electrical stimulation of the track of the vagus nerve beneath the anterolateral skin of the neck (GammaCore©) (23). It is established that transcutaneous VNS (tVNS) could effectively treat epilepsy as well as the invasive VNS (15), and noninvasive VNS (nVNS) is confirmed to be effective in treating primary headache (23).

While the mechanism for the beneficial effects of VNS in epilepsy and mental illness has not been definitively determined, various neuro-immune mechanisms have been considered. The neuroimmunomodulation loop (cholinergic anti-inflammatory pathway) was identified. The release of acetylcholine following the activation of the vagus nerve by pharmacological/electrical stimulation impedes the LPS-induced production of systemic pro-inflammatory cytokines in human macrophages (24). In a pilot study, Lerman *et al* (25) showed that nVNS down-regulated the release of inflammatory cytokines, implying that

nVNS may be an effective anti-inflammatory treatment in mental disorders. However, there have been no studies testing whether the up regulation of cytokines found in patients with PTSD can be decreased or modulated by nVNS in humans.

Our study of PACAP, PTSD, and VNS is derived from a large DARPA funded project headed by Dr. J. Doug Bremner. In this thesis, I utilized data and blood samples from this ongoing DARPA project to evaluate the effect of VNS treatment by comparing PACAP concentration across numerous time points among participants with PTSD or healthy participants that were randomized to either nVNS treatment or sham stimulation (control).

METHODS

Overview

This study was part of the project, *Closed Loop Vagal Stimulation in Patients with Posttraumatic Stress Disorder* funded by DARPA BAA-15-35 (phases 1 and 2, J. Doug Bremner, Principal investigator), which aimed to develop the fundamental physiological understanding required for ultimately improving the quality of life for patients with PTSD through feedback-controlled VNS. This project was a parallel study, in which VNS treatment or sham stimulation were randomly conducted in both patients with PTSD and healthy participants. The study was reviewed and approved by the Emory Institutional Review Board (IRB00091171), ClinicalTrials.gov Identifier: NCT02992899.

Eligibility Criteria

a. Healthy participants: Subjects aged 18-70 with a history of exposure to psychological trauma, but who did not meet criteria for PTSD or other major mental disorder as determined by the Structured Clinical Interview for DSM-5 (SCID) interview for PTSD.

b. Patients with PTSD: Subjects aged 18-70 who had a history of psychological trauma and met criteria for PTSD defined by DSM-5.

Exclusion Criteria

Subjects were excluded with: 1) positive pregnancy test; 2) meningitis; 3) traumatic brain injury; 4) neurological disorder or organic mental disorder; 5) history of loss of consciousness greater than one minute; 6) alcohol abuse or substance abuse or dependence based on the SCID within the past 12 months; 7) current or lifetime history of schizophrenia,

schizoaffective disorder, or bulimia, based on the SCID; 8) a history of serious medical or neurological illness, such as cardiovascular, gastrointestinal, hepatic, renal, neurologic or other systemic illness; 9) evidence of a major medical or neurological illness on physical examination or as a result of laboratory studies (CBC, BUN, creatinine, blood sugar, electrolytes, liver and thyroid function tests, urinalysis, and EKG); 10) active implantable device (i.e. pacemaker); 11) carotid atherosclerosis; and 12) cervical vagotomy. Women were counseled about the risks of pregnancy during the course of the study.

Recruitment

Subjects were recruited from newspaper advertisements, online websites, and/or fliers.

Baseline Assessments

Each subject was provided written consent, after which they would undergo a baseline psychological and health assessment. The health assessment included sociodemographic factors (age, sex, race/ethnicity, marital status, education level) and clinical information (current medications, medical history, etc.) assessed using standardized questions, and the baseline psychological assessment was using structured interviews and questionnaires, which measured PTSD symptoms (PCL-C), traumatic experiences (ETI, LTI), dissociative symptoms (CADSS), life stress (PSS), depressive symptoms (HAM-D, BDI-II), anxiety symptoms (HAMA), anger expression (STAXI-II), hostility (CMHS), sleep quality (Global PSQI), mindfulness (CAMS-R), social support (ESSI), and physical activity (Baecke Questionnaire). Enrolled patients with PTSD also underwent an initial screening and a Structured Clinical Interview for DSM-IV (SCID) to ensure eligibility. The SCID is the

most widely used instrument for the establishment of the psychiatric diagnosis. There was a total of 40 participants after the baseline assessments.

VNS administration

The "VNS" group was administered VNS using the electroCore© GammaCore-S non-invasive VNS device. The intensity of the stimulus (i.e. the current amplitude) was adjusted by the user (roll switch, scale from 0-5), to the maximum tolerable level to ensure VNS without causing excessive pain (typically 10-30 V). The burst frequency was 5 kHz, and the envelope frequency was 25 Hz. These were the standard frequency settings that electroCore© had demonstrated to be most effective in capturing the vagus nerve based on evoked potential studies. Figure 1 provides an overview of the paradigm and timeline for the study. The duration of VNS/sham delivery was 2 minutes, and the beginning of VNS treatment or sham stimulation was immediately after the subject heard each traumatic script at time points 3, 4, 9, 10, 13, 14, 16, 17, 20 and 21. At time point 5 and 6, the beginning of VNS treatment or sham stimulation coincided with initiation of acquisition of the PET scan, which was 90 seconds in duration; followed an additional 8 minutes, and then a second VNS delivery was administered, in conjunction with another scan. Each subject in the "sham stimulation" group underwent the action of administering the intervention, but the device was programmed to delivered power with a lower amplitude and very low frequency (0.2 Hz stepped pattern, almost direct current). The stimulators provided by electroCore© including analog trigger outputs that could provide timing information on when the stimulus was being delivered.

The procedure for Days 2 and 3 followed the same general procedure except no scans were obtained (see below).

Procedures

The protocol follows established methods which have been found to effectively elicit trauma-related stress responses (Figure 1). Prior to the stress procedure, subjects were asked to prepare two scripts of the most stressful events s/he experienced. The scripts were transcribed, edited to last 120 seconds each, and recorded by a research associate in a normal voice, in the first person, present tense.

Day 1 emotional stress. After the initial set up and rest period, subjects rested for 30 min. Next, subjects underwent a series of listening exercises while lying in the HR PET scanner with physiologic monitors attached. The PET scan started with the first "event" at each time point, which could be the neutral script (at time point 1, 2, 7, 8, 11 and 12) or the trauma script (at time point 3, 4, 9, 10, 13, 14) or the VNS or sham stimulation (at time point 5 and 6), and lasts for 8 minutes. After an initial baseline period of resting, the subjects then listened to two neutral 120-second scripts with scans. These neutral scripts were not accompanied by either VNS or sham. Next, the subjects were exposed to playback of two 60-second trauma scripts. Immediately following each trauma script, the subject received either VNS or sham stimulation. The next scanning session did not include a script but each of the two scans were immediately followed by either a VNS or sham stimulation. The next two scans started with two neutral scripts without either VNS or sham stimulation. The next block was a set of trauma scripts. As in the previous trauma script block, each trauma script was followed by a scan and either VNS or sham stimulation. This was followed by another block of two neutral scripts and scans. And finally, a block of two trauma scripts, in which each trauma script with a scan, and followed by either VNS or sham stimulation.

The subject then was outfitted with ambulatory monitors to measure multiple peripheral physiological signals representative of cardiac electrophysiology (electrocardiogram

(ECG)), hemodynamics (i.e., non-invasive blood pressure, impedance cardiography (ICG), photoplethysmogram (PPG)), chest-wall vibrations (seismocardiography (SCG)), respiration, and skin conductance (electrodermal activity (EDA)) to account for physical activity-based changes. Blood was drawn at the following time point: baseline, 4, 6, 8, 10, 12 and 14 (total 194.5cc).

Days 2 and 3, mental stress. On the second and third day, each subject wore ambulatory, comprehensive physiological monitoring systems that measured a range of signals related to autonomic function. Stressful stimulations were performed twice per day at intervals to induce external stress. During the subject's stay, acute mental stressors were introduced. The first of these was a public speech task that was followed by a VNS or sham stimulation. Next was a mental arithmetic task that was again followed by VNS or sham stimulation. Finally, the subject rested for 90 minutes and was given an additional stimulation with either VNS or sham. This procedure was repeated on the third day. Blood was drawn in the lab before the tasks and immediately after the last treatment (total 57cc per day).

Biomarker Measurement

We performed multiplex assays to measure both pro-inflammatory and anti-inflammatory cytokines, as well as other related key blood biomarkers, and traditional ELISA to measure the PACAP concentration in the peripheral blood (Table 3). Multiplex kits were purchased from Meso Scale Discovery. Human PACAP ELISA kits were purchased from LifeSpan BioSciences. All experimental operations were in accordance with standard protocols. R^2s of the standard curves for each plate were greater than 0.999.

Statistical methods

Psychological scales were scored or ranked based on the corresponding guides. ANOVA tests were used to compare the demographic characteristics across the VNS treatment or sham stimulation group among patients with PTSD and healthy participants. For baseline analysis, we built several linear regression models to measure the association between biomarker concentration and psychological scores or PTSD status. Due to the small sample size, we chose to recode some categorical variable to keep the models simple and justifiable. For the race, we coded the White / Caucasian as the reference group, keeping Black / African American unchanged, and classified other races as one group called "Others". Education level was dichotomized to Associate's degree or lower, and Bachelor's degree or higher (reference group). In our study population, none of the subjects was underweight (BMI<18.5). Therefore, we decided to treat BMI as a continuously variable in the following analyses. ANOVA test and multivariate linear regression models were used for the baseline analysis. Linear mixed models were used for the longitudinal analysis. All statistical analyses were done using SAS 9.4.

Models used in the analyses were listed below:

Baseline level test:

Model 1: $log(PACAP) = \beta_0 + \beta_1 PTSD$

Model 2: $log(PACAP) = \beta_0 + \beta_1 PTSD + \beta_2 Sex + \delta_1 PTSD * Sex$

Model 3: $log(PACAP) = \beta_0 + \beta_1 PTSD + \beta_2 Sex + \gamma_1 Age + \gamma_2 BMI + \gamma_3 Race + \gamma_4 Education$

Model 4: $log(PACAP) = \beta_0 + \beta_1 PTSD + \beta_2 Sex + \gamma_1 Age + \gamma_2 BMI + \gamma_3 Race + \gamma_4 Education + \delta_1 PTSD * Sex$

Model 5: $Scores = \beta_0 + \beta_1\, log(PACAP) + \gamma_1 Sex + \gamma_2 Age + \gamma_3 BMI + \gamma_4 Race + \gamma_5 Education$

Model 6: $Dich\_Scores = \beta_0 + \beta_1\, log(PACAP) + \gamma_1 Sex + \gamma_2 Age + \gamma_3 BMI + \gamma_4 Race + \gamma_5 Education$

Longitudinal Analysis:

Model 7: $log(PACAP_{ij}) = \beta_{0i} + b_{0i} + \beta_1 Treat_{ij} + \beta_2 PTSD_{ij} + \beta_3 Time1_{ij} + \beta_4 Time2_{ij} + \beta_5 Time3_{ij} + \gamma_1 Age_{ij} + \gamma_2 BMI_{ij} + \gamma_3 Race_{ij} + \gamma_4 Education_{ij} + \gamma_5 Sex_{ij} + \delta_1 Treat_{ij} * Time1_{ij} + \delta_2 PTSD_{ij} * Time1_{ij} + \delta_3 Treat_{ij} * Time2_{ij} + \delta_4 PTSD_{ij} * Time2_{ij} + \delta_5 Treat_{ij} * Time3_{ij} + \delta_6 PTSD_{ij} * Time3_{ij} + e_{ij}$

Model 8: $log(PACAP_{ij}) = \beta_{0i} + b_{0i} + \beta_1 PTSD_{ij} + \beta_2 Sex_{ij} + \beta_3 Time1_{ij} + \beta_4 Time2_{ij} + \beta_5 Time3_{ij} + \gamma_1 Age_{ij} + \gamma_2 BMI_{ij} + \gamma_3 Race_{ij} + \gamma_4 Education_{ij} + \delta_1 PTSD_{ij} * Time1_{ij} + \delta_2 Sex_{ij} * Time1_{ij} + \delta_3 PTSD_{ij} * Time2_{ij} + \delta_4 Sex_{ij} * Time2_{ij} + \delta_5 PTSD_{ij} * Time3_{ij} + \delta_6 Sex_{ij} * Time3_{ij} + e_{ij}$

Model 9: $log(PACAP_{ij}) = \beta_{0i} + b_{0i} + \beta_1 Treat_{ij} + \beta_2 Sex_{ij} + \beta_3 Time1_{ij} + \beta_4 Time2_{ij} + \beta_5 Time3_{ij} + \gamma_1 Age_{ij} + \gamma_2 BMI_{ij} + \gamma_3 Race_{ij} + \gamma_4 Education_{ij} + \delta_1 Treat_{ij} * Time1_{ij} + \delta_2 Sex_{ij} * Time1_{ij} + \delta_3 Treat_{ij} * Time2_{ij} + \delta_4 Sex_{ij} * Time2_{ij} + \delta_5 Treat_{ij} * Time3_{ij} + \delta_6 Sex_{ij} * Time3_{ij} + e_{ij}$

Biomarker Correlation:

Model 10: $log(Biomarker) = \beta_0 + \beta_1\, log(PACAP) + \gamma_1 Sex + \gamma_2 Age + \gamma_3 BMI + \gamma_4 Race + \gamma_5 Education$

RESULTS

A total of 36 subjects who completed or at least partly completed the trauma tasks were involved in these analyses (Figure 2). Subject groups were similar in age, body mass index, race, education level and marital status (Table 1). For patients with PTSD, only females received VNS treatment (Fisher's Exact p = 0.045), and the gender proportion in the other groups was similar. The average age of this population was 30.33 (SD = 9.03), and the average BMI was 26.59 (SD = 5.60). Among all the subjects, 21(58.3%) were female, 18(50%) White / Caucasian, 21(58.3%) had a Bachelor's or higher degree, and 24(66.7%) had never married.

We also assessed the subjects' psychological and physical functions at baseline and tested whether the scores followed a normal distribution (Table 2 & Appendix Figure 1). When adjusted for potential confounders, the PTSD Checklist score (p = 0.0004), Global PSQI score (p = 0.0295), STAXI-II subscale: Anger expression-out score (p=0.0304) and Perceived Stress Scale score (p=0.0436) were significantly, positively associated with subjects' PTSD diagnosis by CAPS and SCID. The Hamilton Anxiety score (p=0.0585) and Hamilton Depression score (p=0.0737) were suggestive of a positive association with subjects' PTSD diagnosis at an alpha level of 0.1.

We used ELISA to measure the blood levels of PACAP at baseline, and the end of the trauma procedure at the 1st, 2nd and 3rd day. The average PACAP concentration at baseline was 83.53 (SD = 38.2). After the total three days' stress tasks and stimulation (VNS treatment or sham stimulation), the average of the PACAP concentration at baseline and at the end of the last intervention increased to 90.21 (SD = 38.72). Since the distribution of PACAP concentration did not follow a normal distribution, we used log-transformed biomarker concentrations in all of our subsequent analyses.

Baseline Level Test

We examined the association of PACAP concentration with PTSD status at baseline (Figure 3). The average log-transformed PACAP concentration in the patients with PTSD was higher than in the healthy participants, however, this difference was not significant (β = 0.09, 95%CI: (-0.27, 0.45)). After adjusting for the potential covariates, the average log-transformed PACAP concentration was still not significantly different among participants with and without PTSD (β = 0.13, 95%CI: (-0.26, 0.52)). Considering the sex-specific association of PACAP blood levels with PTSD diagnosis, we tested the baseline association of PACAP concentration and PTSD status in males and females separately. Both in the female and male group, the PACAP concentration was not significantly different between patients with PTSD and participants without PTSD. The average log-transformed PACAP concentration was lower among male patients with PTSD than healthy male participants in the crude model (β = -0.12, 95%CI: (-0.78, 0.52)), but the direction was inverted after controlling for potential covariates (β = 0.12, 95%CI: (-0.70, 0.95)). In females, the average log-transformed PACAP concentration was higher among patients with PTSD than among the healthy participants before and after controlling for potential covariates (crude β = 0.16, 95%CI: (-0.35, 0.67); adjusted-β = 0.20, 95%CI: (-0.53, 0.93)).

Next, we examined the association between baseline PACAP concentration and scores of psychological and functional scales (Figure 4). For this analysis, if the psychological scores followed a normal distribution, we examined the association in log-transformed PACAP blood levels with the raw scores. If the psychological scores did not follow a normal distribution, we used either the natural cut-offs of the scores or dichotomized the scores by the median value. However, using the natural cut-offs of the scores, the models either did

not converge or the resulting confidence intervals were artificially large. As a result, we decided to dichotomize the scores that did not follow a normal distribution by the median and examined the associations (Figure 4B, 4D). The baseline log-transformed PACAP concentration was positively correlated with scores of PTSD symptoms (PCL-C), early traumatic experiences (ETI), depressive symptoms (HAM-D), anger state, general trait expression index, and control-in trend (STAXI-II subscales: Current State, General Trait, Expression Index and Control-In), and social support (ESSI). Conversely, the baseline PACAP concentration had a negative correlation with the scores of hostility (CMHS), life stress (PSS), anger expression-in (STAXI-II subscales: Expression-In), sports and work activity (Baecke Questionnaire subscale: Sport & Work). Except for the raw scores of ESSI and Baeche Questionnaire Sport Index, none of the associations between baseline PACAP concentration and the scores of psychological and functional scales were significant (Figure 4). However, since the distribution of the raw scores of ESSI and Baeche Questionnaire Sport Index did not follow the normal distribution, we also tested the association of the dichotomous scores and the log-transformed PACAP concentration, and the association was not significant (Figure 4D).

Longitudinal Analysis

In the next step, we examined how PACAP blood levels changed over time under the trauma and stressful tasks (Table 3). Since the experimental paradigm of trauma and stressful tasks was found to increase the subjects' stress level, we hypothesized that PACAP concentration was related to the stress level, and that PACAP concentration would increase after the series of experimental stressful tasks.

Controlling for subjects' age, sex, BMI, race, education level, and considering the heterogeneity across individuals, the point estimates for the increase in PACAP was greater in the sham then the VNS at every time point. In our models, we tested for significant differences between each of the subgroups. Specifically, among the healthy subjects received sham treatment, there was an increase in PACAP of 17% (95%CI: (1.00, 1.36)), 19% (95%CI: (1.03, 1.38)), and 25% (95%CI: (1.07, 1.45)) relative to the baseline concentration at the end of Day 1, Day 2 and Day 3, respectively. Each of these time points indicated a statistically significant increase over baseline ($P<0.05$). Among the patients with PTSD in the sham group, compared with the baseline, the average PACAP concentration increased about 4% (95%CI: (0.87, 1.23)), 12% (95%CI: (0.96, 1.32)) and 12% (95%CI: (0.95, 1.32)) at the end of Day 1, Day 2 and Day 3, respectively. However, none of the changes in the PTSD group were statistically significant. The interaction between PTSD diagnosis and time was not significant ($p>0.05$).

Among subjects who received VNS treatment, the rise in PACAP at each time point was diminished relative to the increase in the sham group at the corresponding time point (Table 3). The PACAP blood levels in the healthy subjects significantly increased 12% (95% CI: (1.00, 1.26)) to the baseline at the end of Day 2. Except for this time point, none of the subgroups who received the VNS treatment (healthy subjects or patients with PTSD) had a statistically significant increase in PACAP blood levels during the 3 days. We also examined if VNS treatment varied with the change of PACAP blood levels over time, but the interaction between VNS treatment and time was not significant (p values for the interaction terms in (0.2 – 0.5)). Although the VNS treatment effect was not significant in any of the groups, the results indicated that VNS treatment might depress the effect of trauma or stressful tasks to the PACAP concentration (Table 3; Appendix figure 2A).

Sex Differences in Trauma Procedure and VNS Treatment

We next examined the possible role of sex in PACAP changes with the stress paradigm, controlling for age, BMI, race, education level, and considering the heterogeneity across individuals (Table 4). Among the healthy male subjects, the average PACAP blood levels significantly increased by 15% (95%CI: (1.02, 1.30)), 13% (95%CI: (1.01, 1.27)) and 21% (95%CI: (1.07, 1.36)) relative to the baseline concentration at the end of Day 1, Day 2 and Day 3, respectively. Among the male subjects with PTSD, the average PACAP blood levels also increased, but increase was not statistically significant (although the number of subjects was only 3 this group). Among the healthy female subjects, the average PACAP concentration increased less than in the healthy male subjects, and the only statistically significant increase was at the end of the second day (16% to the baseline, 95%CI: (1.02, 1.34)). Among female patients with PTSD none of the changes in PACAP were statistically significant. The PACAP concentration dropped by 3% relative to the baseline (95% CI: (0.82, 1.15)) at the end of the Day 1. At the end of Day 2 and Day 3, the PACAP concentration increased insignificantly among the female patients with PTSD, and less than in the healthy female subjects. The results indicated that the PACAP blood levels in healthy participants might be more sensitive to the trauma and stress tasks, and more sensitive among males than that among females (Table 4; Appendix figure 2B). We also found that the VNS treatment diminished the increase in PACAP blood levels both in males and females (Table 5; Appendix figure 2C), although the interaction between VNS treatment and time was still not significant (p values for the interaction terms in (0.4 – 0.75)). In addition, given that our sample had no males with PTSD who were randomized to the VNS treatment group, we did not examine sex-specific effects of VNS treatment by PTSD diagnostic group.

Association with other biomarkers

As described in the previous sections the blood levels of PACAP were related to the trauma procedure. It is well known that stress can cause variations in the blood levels of multiple biomarkers. Therefore, we tested the association between the blood levels of PACAP and other stress-related biomarkers. We used multiplex assays to measure biomarker blood levels in all groups, for both baseline and all time points. We also tested the normality for the distribution of each biomarker (Table 6). As for PACAP, these other biomarkers were measured in the same individuals longitudinally. Therefore, the number of samples for a given group may exceed the total number of individuals in that group (Figure 1, table 6).

Adjusted for age, sex, BMI, race, and education level, the log-transformed PACAP concentration was significantly, positively associated with the log-transformed concentration of IFN-γ ($\beta = 0.14$, $p=0.022$) and Ghrelin ($\beta = 0.15$, $p=0.05$) and significantly, negatively correlated with IL6 ($\beta = -0.13$, $p=0.048$), IL13 ($\beta = -0.25$, $p=0.006$), and IL5 ($\beta = -0.27$, $p=0.013$) (Table 7). The blood levels of these related biomarkers at each measured time points were plotted in Figure 3.

DISCUSSION

In the central nervous system, PACAP is involved in a variety of pathological and physiological processes, including those participating in advanced brain function, mood, behavioral regulation, and neuroprotection (26, 27). Lehmann et al. (28) studied PACAP-deficient mice, and reported that indicated that PACAP appeared to be specific for mediating HPA activation only in psychological stress. However, there is still no evidence to show how PACAP changes in humans undergoing stressful tasks. Our study is the first to demonstrate that PACAP levels in peripheral blood increased constantly after the trauma and stress tasks. This confirms that PACAP plays an important role in the CNS response to chronic stress.

We hypothesized that VNS may dampen the increase in PACAP due to traumatic recall or other psychological stressors. As a preliminary study, we examine this in several ways. At each time point after traumatic scripts and other stressful events, the increasing PACAP was greater among the sham treated versus the VNS treated subjects. However, our specific interaction term for the treatment effect on change of PACAP was not significant.

In our study, we found that PACAP levels in blood were significantly, negatively correlated with IL-6, IL-13, and IL-5 (Table 3). These correlations may indicate the pathophysiological mechanisms in which PACAP coordinates with other cytokines in peripheral stress. As a peptide with anti-inflammatory function, PACAP was found to regulate the function of inflammatory cells through specific receptors, affecting innate immunity and adaptive immunity (29). PACAP can directly inhibit the production of pro-inflammatory cytokines (such as IL-6) by acting on macrophages (29). Our finding of the negative correlation between PACAP and IL-6 suggest that PACAP may have a counter

regulatory effect of hyper inflammation associated with PTSD. Conversely, IL-5 and IL-13 are mainly TH2 type helper cell cytokines. Low spontaneous immune activity (low levels of IL-13 and IL-5) were found in children from families with high psychological stress (30). Hence it is possible that the negative correlation between PACAP and IL13 and IL5 may indicate that PACAP is involved in the promotion or maintenance of decreased levels of these cytokines, which are also linked to asthma.

On the other hand, we found that the blood levels of PACAP were significantly positively associated with the levels of IFN-γ and ghrelin. There is inconsistency in the literature as to whether PACAP is playing a beneficial role acting as a neuro- hormone in the circulation to counteract the negative consequences of stress (29), or if it is harmful since administrating PACAP after stress stimulation increases and prolongs anxiety (17). Our study is the first in humans to examine the correlation between PACAP and these other soluble mediators that are increased in chronic stress. Since there was a positive association between PACAP and ghrelin concentration, studies of the dual effect of ghrelin in modulating anxiety-related behaviors may shed light on the potential dual effect of PACAP. Spencer *et al.* suggested that ghrelin could increase anxiety under non-stressed conditions, and decrease anxiety after acute stress (31). It is possible that the blood levels of PACAP increased after the stressful tasks might increase ghrelin and thus have a beneficial effect by preventing excessive anxiety. We are currently examining the relationship between PACAP, ghrelin, and perceived psychological stress during the paradigm. The function of IFN-γ in stress is still unclear, but most research (32-34) indicates that IFN-γ synergizes with stress and may be involved in the immune abnormalities in development of psychological depression associated with chronic stress. The mechanism underlying the correlation between IFN-γ and PACAP remains to be investigated in future studies.

Since the isolation and identification of PACAP in 1989, research has demonstrated the relationship between PACAP and mental disorders, such as schizophrenia (35), major depression (36), and post-traumatic stress disorder (13). In our study, we found that after controlling for potential confounders, there is a higher blood level of PACAP in patients with PTSD than healthy subjects at baseline, although the difference was not significant. Ressler *et al.* (13) observed a sex-specific association with female PTSD diagnosis. However, in our study, we did not find such a sex disparity in the baseline association between PTSD diagnosis and PACAP levels (Figure 2), despite using the log-transformed PACAP or dichotomized PACAP blood level (low vs. high, results not showed). This might be due to the small sample size and sex imbalance in our subjects with PTSD (3 males vs. 9 females).

We observed that the VNS treatment may moderate the trauma and stress task effect to PACAP blood level in the healthy subjects but not in the patients with PTSD, though the effect was not significant. We also noticed there is a sex disparity in the VNS treatment effect. The PACAP blood level in females may be more sensitive to the VNS treatment than males, especially in Day 2 and Day 3. Nevertheless, small sample size especially in stratified analysis limits the interpretation of sex specific changes in PACAP. In the next study phase, we will combine more relevant cytokines and the brain scan images to determine the effects of VNS treatment. Even though the change of PACAP blood levels could directly reflect the effect of VNS treatment, because of the lack of male patients with PTSD in our study receiving VNS treatment, it should not be asserted at this stage that VNS treatment is better for women than for men, or better for healthy subjects than for patients with PTSD.

Our study has some limitations. Firstly, we have only 12 patients with PTSD in our study, and all the three male patients were randomly assigned to the sham stimulation group.

Due to this small sample size and the fact that none of the PTSD male subjects received the VNS treatment, we cannot evaluate the effect of VNS treatment on PACAP concentrations among PTSD males. Moreover, as gender may be an effect modifier of PACAP concentrations and PTSD, this interaction could not be evaluated within the VNS treatment group. Prior work has suggested that blood levels of PACAP are associated with PTSD diagnosis among females and stress-regulation pathways may vary between men and women. Secondly, PACAP is involved in circadian rhythm regulation (37-39). Since our study lasted for three days for each subject and blood draws were at different times each day, there may be concerns that the blood levels of PACAP would naturally change throughout the day. However, we did not find any published reports of PACAP levels changing in the peripheral blood, and blood draws on day two and three were in the morning. Thirdly, we measured PACAP blood levels at only four time points. Since we did not measure the PACAP blood levels at the baseline on the second and the third days, we were unable to directly see the changes in PACAP on the second and the third days; consequently, we may be missing important information.

CONCLUSION & FUTURE DIRECTIONS

We identified that trauma or stressful tasks were associated with increased PACAP concentration in the peripheral blood. My thesis is the first report of the PACAP blood levels in humans undergoing a trauma recall paradigm. However, the association in VNS treatment to the PACAP concentration level still needs to be established in the Phase 2 study with more subjects. Combined with some correlated inflammatory cytokines, PACAP might be a biomarker to show or predict the treatment effect of VNS to PTSD or the other mental disorders.

One of the goals of this VNS study is to exam the modulation of sympathetic and parasympathetic regulation of cardiovascular dynamics, and PACAP is well-positioned to participate in this mechanism. While many of the details in humans have not been examined, animal models suggest that PACAP enhances excitability of parasympathetic cardiac ganglion (14). Thus, a future direction of our study is to examine individual differences in PACAP induction by trauma recall or stress paradigms in relation to changes in heart rate or other cardiovascular parameters.

REFERENCES

1. National Institute of Mental Health. Post-Traumatic Stress Disorder 2019 [Available from: https://www.nimh.nih.gov/health/topics/post-traumatic-stress-disorder-ptsd/index.shtml.

2. Gradus JL. Epidemiology of PTSD. National Center for PTSD (United States Department of Veterans Affairs). 2007.

3. Lipov EG, Kevin Burkhardt, and Jessica C. Smith. A novel application of stellate ganglion block: preliminary observations for the treatment of post-traumatic stress disorder. Military medicine. 2012;177.2 (2012):125.

4. U.S. Department of Veterans Affairs. PTSD: National Center for PTSD 2019 [Available from: https://www.ptsd.va.gov/understand/common/common_adults.asp.

5. Kessler RC, Aguilar-Gaxiola S, Alonso J, Benjet C, Bromet EJ, Cardoso G, et al. Trauma and PTSD in the WHO world mental health surveys. 2017;8(sup5):1353383.

6. Yehuda R, Hoge CW, McFarlane AC, Vermetten E, Lanius RA, Nievergelt CM, et al. Post-traumatic stress disorder. Nature Reviews Disease Primers. 2015;1:15057.

7. Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CBJAogp. Posttraumatic stress disorder in the National Comorbidity Survey. 1995;52(12):1048-60.

8. Va BJF, Health ATSoM. Posttraumatic stress disorder in the National Comorbidity Survey. 2013;10:22.

9. Bourla A, Mouchabac S, El Hage W, Ferreri FJEjop. e-PTSD: An overview on how new technologies can improve prediction and assessment of Posttraumatic Stress Disorder (PTSD). 2018;9(sup1):1424448.

10. Leonard BEJEP. The HPA and immune axes in stress: the involvement of the serotonergic system. 2005;20:S302-S6.

11. Daskalakis NP, Cohen H, Nievergelt CM, Baker DG, Buxbaum JD, Russo SJ, et al. New translational perspectives for blood-based biomarkers of PTSD: from glucocorticoid to immune mediators of stress susceptibility. 2016;284:133-40.

12. Passos IC, Vasconcelos-Moreno MP, Costa LG, Kunz M, Brietzke E, Quevedo J, et al. Inflammatory markers in post-traumatic stress disorder: a systematic review, meta-analysis, and meta-regression. 2015;2(11):1002-12.

13. Ressler KJ, Mercer KB, Bradley B, Jovanovic T, Mahan A, Kerley K, et al. Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. 2011;470(7335):492.

14. Starr ER, Margiotta JF. PACAP modulates distinct neuronal components to induce cell-specific plasticity at central and autonomic synapses. Pituitary Adenylate Cyclase Activating Polypeptide—PACAP: Springer; 2016. p. 83-107.

15. Ergang P, Vodička M, Soták M, Klusoňová P, Behuliak M, Řeháková L, et al. Differential impact of stress on hypothalamic–pituitary–adrenal axis: gene expression changes in Lewis and Fisher rats. 2015;53:49-59.

16. Masuo Y, Matsumoto Y, Tokito F, Tsuda M, Fujino MJBr. Effects of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) on the spontaneous release of acetylcholine from the rat hippocampus by brain microdialysis. 1993;611(2):207-15.

17. King SB, Lezak KR, O'Reilly M, Toufexis DJ, Falls WA, Braas K, et al. The effects of prior stress on anxiety-like responding to intra-BNST pituitary adenylate cyclase activating polypeptide in male and female rats. 2017;42(8):1679.

18. Koek RJL, Tinh Theranostic pharmacology in PTSD: Neurobiology and timing. 2018.

19. Koek RJ, Roach J, Athanasiou N, van t Wout-Frank M, Philip NSJPiN-P, Psychiatry B. Neuromodulatory treatments for post-traumatic stress disorder (PTSD). 2019.

20. Edwards CA, Kouzani A, Lee KH, Ross EK, editors. Neurostimulation devices for the treatment of neurologic disorders. Mayo Clinic Proceedings; 2017: Elsevier.

21. Noble LJ, Gonzalez I, Meruva V, Callahan KA, Belfort BD, Ramanathan K, et al. Effects of vagus nerve stimulation on extinction of conditioned fear and post-traumatic stress disorder symptoms in rats. 2017;7(8):e1217.

22. Giordano F, Zicca A, Barba C, Guerrini R, Genitori LJE. Vagus nerve stimulation: Surgical technique of implantation and revision and related morbidity. 2017;58:85-90.

23. Mertens A, Raedt R, Gadeyne S, Carrette E, Boon P, Vonck KJEromd. Recent advances in devices for vagus nerve stimulation. 2018;15(8):527-39.

24. Zila I, Mokra D, Kopincova J, Kolomaznik M, Javorka M, Calkovska AJPr. Vagal-immune interactions involved in cholinergic anti-inflammatory pathway. 2017;66.

25. Lerman I, Hauger R, Sorkin L, Proudfoot J, Davis B, Huang A, et al. Noninvasive transcutaneous vagus nerve stimulation decreases whole blood culture-derived cytokines and chemokines: a randomized, blinded, healthy control pilot trial. 2016;19(3):283-90.

26. Hashimoto H, Shintani N, Tanida M, Hayata A, Hashimoto R, Baba AJCpd. PACAP is implicated in the stress axes. 2011;17(10):985-9.

27. Mustafa T. Pituitary adenylate cyclase-activating polypeptide (PACAP): a master regulator in central and peripheral stress responses. Advances in Pharmacology. 68: Elsevier; 2013. p. 445-57.

28. Lehmann ML, Mustafa T, Eiden AM, Herkenham M, Eiden LEJP. PACAP-deficient mice show attenuated corticosterone secretion and fail to develop depressive behavior during chronic social defeat stress. 2013;38(5):702-15.

29. Delgado M, Abad C, Martinez C, Juarranz MG, Leceta J, Ganea D, et al. PACAP in immunity and inflammation. 2003;992(1):141-57.

30. Carlsson E, Frostell A, Ludvigsson J, Faresjö MJTjoI. Psychological stress in children may alter the immune response. 2014;192(5):2071-81.

31. Spencer SJ, Xu L, Clarke MA, Lemus M, Reichenbach A, Geenen B, et al. Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. 2012;72(6):457-65.

32. Curtin NM, Boyle NT, Mills KH, Connor TJJB, behavior,, immunity. Psychological stress suppresses innate IFN-γ production via glucocorticoid receptor activation: Reversal by the anxiolytic chlordiazepoxide. 2009;23(4):535-47.

33. Litteljohn D, Cummings A, Brennan A, Gill A, Chunduri S, Anisman H, et al. Interferon-gamma deficiency modifies the effects of a chronic stressor in mice: implications for psychological pathology. 2010;24(3):462-73.

34. Litteljohn D, Rudyk C, Razmjou S, Dwyer Z, Syed S, Hayley SJNi. Individual and interactive sex-specific effects of acute restraint and systemic IFN-γ treatment on neurochemistry. 2017;102:95-104.

35. Hashimoto R, Hashimoto H, Shintani N, Chiba S, Hattori S, Okada T, et al. Pituitary adenylate cyclase-activating polypeptide is associated with schizophrenia. 2007;12(11):1026.

36. Hashimoto R, Hashimoto H, Shintani N, Ohi K, Hori H, Saitoh O, et al. Possible association between the pituitary adenylate cyclase-activating polypeptide (PACAP) gene and major depressive disorder. 2010;468(3):300-2.

37. Cagampang FRA, Piggins HD, Sheward WJ, Harmar AJ, Coen CWJBr. Circadian changes in PACAP type 1 (PAC1) receptor mRNA in the rat suprachiasmatic and supraoptic nuclei. 1998;813(1):218-22.

38. Fukuhara C, Suzuki N, Matsumoto Y, Nakayama Y, Aoki K, Tsujimoto G, et al. Day-night variation of pituitary adenylate cyclase-activating polypeptide (PACAP) level in the rat suprachiasmatic nucleus. 1997;229(1):49-52.

39. Kallo I, Kalamatianos T, Piggins H, Coen CWJJon. Ageing and the diurnal expression of mRNAs for vasoactive intestinal peptide and for the VPAC2 and PAC1 receptors in the suprachiasmatic nucleus of male rats. 2004;16(9):758-66.

TABLES

Table 1. Demographic Characteristics by PTSD Diagnosis and Treatment Groups

Table 1. Demographic Characteristics by PTSD and Treatment Groups

	Patients with PTSD			Healthy Subjects			Total (n = 36)
Variables	**VNS (n = 7)**	**sham (n = 5)**	**Total (n = 12)**	**VNS (n = 13)**	**sham (n = 11)**	**Total (n = 24)**	
Age (Mean ± SD)	28.29 ± 7.34	32.20 ± 7.60	29.92 ± 7.38	28.77 ± 8.26	32.64 ± 11.60	30.54 ± 9.90	30.33 ± 9.03
BMI (Mean ± SD)	23.57 ± 6.89	30.57 ± 4.61	26.49 ± 6.83	26.66 ± 5.30	26.61 ± 4.95	26.63 ± 5.03	26.59 ± 5.60
Sex (N (%))							
Male	0 (0.0)	3 (60.0)	3 (25.0)	8 (61.5)	4 (36.4)	12 (50.0)	15 (41.7)
Female	7 (100)	2 (40.0)	9 (75.0)	5 (38.5)	7 (63.6)	12 (50.0)	21 (58.3)
Race (N (%))							
White / Caucasian	3 (42.9)	3 (60.0)	6 (50.0)	6 (46.2)	6 (54.5)	12 (50.0)	18 (50.0)
African American	4 (57.1)	1 (20.0)	5 (41.7)	3 (23.1)	2 (18.2)	5 (20.8)	10 (27.8)
American Indian / Alaska Native	1 (14.3)	1 (20.0)	2 (16.7)	0 (0.00)	0 (0.0)	0 (0.00)	2 (5.6)
Asian	0 (0.0)	1 (20.0)	1 (8.3)	2 (15.4)	2 (18.2)	4 (16.7)	5 (13.9)
Native Hawaiian / Pacific Islander	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.00)	0 (0.0)	0 (0.00)	0 (0.0)
Multiracial	1 (14.3)	0 (0.0)	1 (8.3)	0 (0.00)	1 (9.1)*	1 (4.2)	2 (5.6)
Other	0 (0.0)	0 (0.0)	0 (0.0)	2 (15.4)	0 (0.0)	2 (8.3)	2 (5.6)
Education Level (N (%))							
High school - graduate	0 (0.0)	1 (20.0)	1 (8.3)	1 (7.7)	0 (0.0)	1 (4.2)	2 (5.6)
College -not complete	3 (42.9)	1 (20.0)	4 (33.3)	3 (23.1)	1 (9.1)	4 (16.7)	8 (22.2)
Associate's degree	2 (28.6)	0 (0.0)	2 (16.7)	1 (7.7)	2 (18.2)	3 (12.5)	5 (13.9)
Bachelor's degree	2 (28.6)	3 (60.0)	5 (41.7)	5 (38.5)	5 (45.5)	10 (41.7)	15 (41.7)
Master's degree	0 (0.0)	0 (0.0)	0 (0.0)	3 (23.1)	3 (27.3)	6 (25.0)	6 (16.7)
Marital status (N (%))							
Never married	6 (85.7)	2 (40.0)	8 (66.7)	9 (69.2)	7 (63.6)	16 (66.7)	24 (66.7)
Married/ Civil Partnership	0 (0.0)	1 (20.0)	1 (8.3)	3 (23.1)	2 (18.2)	5 (20.8)	6 (16.7)
Divorced / Separated	1 (14.3)	1 (20.0)	2 (16.7)	1 (7.7)	2 (18.2)	3 (12.5)	5 (13.9)
Widowed	0 (0.0)	1 (20.0)	1 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.8)

* This participant reported Multiracial but did not check the races s/he belonged.

Table 2. Description and Statistics of Psychological Scales Scores

Table 2. Psychological Scales Summary

Assessment	Skewness	Kurtosis	Median (Lower Quartile, Upper Quartile)	Normality[a]	Description
Early Trauma Inventory (ETI) (n = 29)	0.886	0.375	5 (3, 8)	0.014	The Early Trauma Inventory is 56-item instrument for measurement of childhood traumatic experiences developed by the applicant and colleagues which has been shown to be reliable and valid. All subjects will be assessed with both the ETI for events before the age of 18 and the Lifetime Trauma Inventory (LTI) for events after the age of 18. The ETI assesses physical, emotional, and sexual abuse, and general traumatic events in childhood, while the LTI assesses a broad range of adult traumatic events. High score indicates more severe trauma the subject experienced before age of 18 (ETI) and in the lifetime (LTI).
Lifetime Trauma Inventory (LTI) (n = 35)	0.974	-0.145	4 (2, 8)	0.001	
Perceived Stress Scale (PSS)* (n = 35)	0.162	-0.601	19 (11, 22)	0.401	The Perceived Stress Scale (PSS) assesses different areas of life stress (e.g., overall stress, financial stress, occupational stress, significant other stress, parental stress, and stress within friendships). Moreover, the PSS has documented validity and reliability, and is short (14 items) and easy to administer. A total score is derived by summing responses to all items, with higher scores indicating greater perceived stress. 10 items, high range is 40, low range is 0. Scoring 3-12 add numbers from items (0-4).
PTSD Checklist (PCL-C)* (n = 32)	0.724	-0.122	31.5 (23, 43.5)	0.062	The PTSD Checklist (civilian version) is a standardized self-report rating scale for PTSD comprising 17 items that correspond to the key symptoms of PTSD. This scale is used for screening individuals for PTSD, for diagnosing PTSD, and for monitoring symptom change during and after treatment. There are two versions of the PCL: military and civilian version. The PCL-C (civilian) asks about symptoms in relation to "stressful experiences," and can be used with any population. The symptoms endorsed may not be specific to just one event, which can be helpful when assessing survivors who have symptoms due to multiple events. Two ways to score this. 1) Add up all the items for a total severity score, or 2) Treat response categories 3-5 (Moderately or above) as symptomatic and responses 1-2 (below

Table 2. Psychological Scales Summary

Assess-ment	Skew-ness	Kurt-osis	Median (Lower Quartile, Upper Quartile)	Norm-ality[a]	Description
					moderately) as non-symptomatic, then us the following DSM criteria for a DX. Symptomatic response to at least 1 "B" item (questions 1-5), Symptomatic response to at least 3 "C" items (questions 6-12), and Symptomatic response to at least 2 "D" items (questions 13-17). DR. BREMNER scoring is 68 - add numbers from items (0-4). Higher score indicates more severe symptoms related to stressful experiences.
BECK DEPRESS-ION INVEN-TORY – II (n = 35)	1.090	0.496	10 (5, 20)	0.002	The Beck Depression Inventory (BDI) is a self-administered 21-item scale which has acceptable sensitivity and specificity with regards to a clinical diagnosis of depression, and also provides a continuous measure of depressive symptoms. It has been used extensively in studies of IHD. We will also assess previous self-reported diagnosis of major depression and previous use of antidepressant medications. LOW =1-10 = normal ups and downs, 11-16 = mild mood disturbance. MODERATE = 17-20 = Borderline clinical depression, 21-30 = moderate depression. SIGNIFICANT = 31-40 = severe depression, Over 40 = extreme depression.
State-Trait Anger Expression Inventory (STAXI-II) (n = 35)					(Spielberg 1991) is a self-report measure of anger expression; specifically, an "Anger-In" scale is computed by summing 8 of the items, and an "Anger-Out" scale is computed by summing the other 8 items. The STAXI has well-documented reliability and validity (Barefoot, Dodge et al. 1989). 57 items, low range is 0, high range is 171. Add item scores (0-3). People with higher scores of each subscales indicate: 1) State Anger: people experienced more intense anger which may be manifested as a desire to scream or break things. 2) Trait Anger: a person's general predisposition to become angry is quicker (vs. slower) 3) Anger Expression-Out: more likely to express their anger outwardly in negative ways either by directing their anger physically toward objects or people, or by using verbal hostility
ANGER: Current State	3.867	18.079	15 (15 , 18)	0.000	
ANGER: General Trait	0.741	0.152	18 (15 , 21)	0.051	
Anger Expression Index	-0.098	-0.937	15 (12 , 17)	0.325	
Anger Expression -Out*	0.432	-0.030	18 (14 , 20)	0.216	
Anger Expression -In	-0.517	-0.729	26 (19.43 , 30)	0.040	

Table 2. Psychological Scales Summary

Assess-ment	Skew-ness	Kurt-osis	Median (Lower Quartile, Upper Quartile)	Norm-ality[a]	Description
Anger Control-Out	-0.546	-0.780	26 (20 , 29)	0.015	such as sarcasm, yelling, or threats. 4) Anger Expression-In: more likely to hold things in or suppress anger when they are angry or furious.
Anger Control-In	0.306	-0.682	31 (21 , 42.7143)	0.494	5) Anger Control-Out: tend to work harder psychologically in monitoring themselves to prevent any explosive manifestations of their anger. 6) Anger Control-In: more often a person attempts to relax, calm down, and reduce angry feelings before they get out of control. 7) Anger Index: the overall estimate, higher score indicates the subject is more likely to express anger either outwardly toward other people, or inwardly toward herself.
Cook-Medley Hostility Scale (CMHS) (n = 35)	-0.134	0.181	69 (66 , 74)	0.637	The Cook-Medley Hostility Scale (CMHS) is a 50-item, true or false self-report hostility scale. It is one of the most widely used self-report hostility measures and taps into the cognitive and affective components of anger more so than behavioral anger. This scale will be used to assess participants' propensity to experience hostility and/or anger, and has satisfactory reliability. 50 items, low range is 0, high range is 50. Flipped items add numbers from items (0-1). Higher score indicates higher hostility of the subject.
Pittsburgh Sleep Quality Index (PSQI)* (n = 34)	0.205	0.265	9 (6 , 10)	0.508	Pittsburgh Sleep Quality Index - self-rated questionnaire which assesses sleep quality and disturbances over a 1-month time interval. (Buysse, Reynolds et al. 1989) The 19 self-rated items are combined to form seven "component" scores, each of which has a range of 0-3 points. The seven component scores are then added to yield one "global" score, with a range of 0-21 points. 0 indicating no difficulty and 21 indicating severe difficulties in all areas.
ENRICH D Social Support Instrument (ESSI) (n = 35)	-1.200	1.103	28 (23 , 32)	0.001	A five-item scale assessing both social and emotional support. The higher the total, the more social support they have. 7 items, high range = 8-yes/no. add numbers from items (0-4)

Table 2. Psychological Scales Summary

Assess-ment	Skew-ness	Kurt-osis	Median (Lower Quartile, Upper Quartile)	Norm-ality[a]	Description
Cognitive and Affective Mindfulness Scale (CAMS-R) (n = 33)	0.020	-0.315	31 (26.5 , 33.5)	0.829	The Revised 12-item Cognitive and Affective Mindfulness Scale (CAMS-R) is an uni-dimensional, 12-item inventory that measures mindfulness during general daily occurrences on four components allegedly needed to reach a mindful state (i.e., attention, awareness, present-focus, and acceptance/nonjudgment). Scoring: Items 2, 6, and 7 are reverse-scored. After appropriate reversals, sum values for items 1 - 12. Higher values reflect greater mindful qualities.
Baecke Questionnaire (n = 35)					Physical activity will be assessed by means of the Baecke Questionnaire of Habitual Physical Activity. This is a 16-question instrument with three sections: work, sports, and non-sports leisure activity. Questions are scored on a five-point Likert scale, ranging from "never" to "always or very often." The scale yields three activity sub-scores, work, sports and non-sports leisure indexes, which can be combined into a total score.
Work Index	-0.874	-0.297	2.5 (2.25, 2.88)	0.000	
Sport Index	0.591	-0.733	2.25 (1.5, 3.25)	0.008	
Leisure Index	0.288	-0.148	2.6 (2.14, 2.86)	0.783	Add item scores (0-3) that have range of 4 responses. Higher score indicates higher physical activity everyday.
Clinician Administered Dissociative States Scale (CADSS) (n = 33)	2.530	5.482	0 (0 , 1)	0.000	The Clinician Administered Dissociative States Scale (CADSS) is a 28-item measure of current "state" level of dissociative symptomatology. The CADSS was developed by the applicant, and there is currently a manuscript in press reporting reliability and validity, sensitivity to change, and other psychometric properties. 23 items, low range 0, high range 92. Add severity in individual items (0-4). Higher score indicates more severe dissociative symptoms.
Hamilton Anxiety (HAMA) (n = 30)	1.576	2.890	4 (0 , 10)	0.000	HAMA is a validated measure of anxiety symptoms with provides a continuous measure of anxiety. (Hamilton 1959) The Ham-A will be used to assess level of anxiety in subjects. Higher score indicates more severe anxiety symptoms.

Table 2. Psychological Scales Summary

Assess-ment	Skew-ness	Kurt-osis	Median (Lower Quartile, Upper Quartile)	Norm-ality[a]	Description
Hamilton Depression (HAM-D) (17 items) (n = 32)	0.793	-0.612	4.5 (0.5 , 10.5)	0.001	HAM-D (Hamilton 1960) is a standardized scale that provides a continuous measure of depressive symptom level which will be administered to study participants. Although the HAM-D form lists 21 items, the scoring is based on the first 17. It generally takes 15-20 minutes to complete the interview and score the results. Eight items are scored on a 5-point scale, ranging from 0 = not present to 4 = severe. Nine are scored from 0-2. SUM the scores from the first 17 items: 0-7 = NORMAL, 8-13 = Mild Depression, 14-18 = Moderate Depression, 19-22 = Severe Depression, > = Very Severe Depression

[a]: P-value is provided by Shapiro-Wilk test for normality. $P < 0.05$ is considered as a violation of the normality.
*: Significantly associated with PTSD diagnosis ($p<0.05$), adjusted for age, sex, BMI, race and education level.

Table 3. Ratio of PACAP concentration to the baseline PACAP concentration over time, grouped by treatment groups & PTSD diagnosis, measured from baseline to the end of Day 3.

Table 3. Ratio of PACAP Concentration to Baseline Over Time, Measures from Baseline to Day 3[a] (by Treatment Groups & PTSD Diagnosis)

(Adjusted-β Estimate (95% Confidence Interval))

	No. of Subjects	Sham Stimulation (n=16)	No. of Subjects	VNS Treatment (n=20)
Healthy Participants				
Baseline	7	ref.	13	ref.
Day1	6	1.17* (1.00, 1.36)	12	1.09 (0.97, 1.22)
Day2	7	1.19* (1.03, 1.38)	13	1.12* (1.00, 1.26)
Day3	6	1.25* (1.07, 1.45)	12	1.11 (0.99, 1.25)
Patients with PTSD				
Baseline	5	ref.	5	ref.
Day1	4	1.04 (0.87, 1.23)	3	0.97 (0.80, 1.16)
Day2	5	1.12 (0.96, 1.32)	4	1.06 (0.89 1.25)
Day3	5	1.12 (0.95, 1.32)	3	1.00 (0.84 1.20)

[a:] Models controlling for age, sex, BMI, race and education level

*Significantly increase over time ($p<0.05$)

Note: No significant interaction between PTSD diagnosis or VNS treatment and Time

Table 4. Ratio of PACAP concentration to the baseline PACAP concentration change over time, grouped by sex and PTSD diagnosis, measured from baseline to the end of Day 3.

Table 4. Ratio of PACAP Concentration to Baseline Over Time, Measures from Baseline to Day 3[a] (by sex & PTSD Diagnosis)

(Adjusted β Estimate (95% Confidence Interval))

	No. of Subjects	Males (n=15)	No. of Subjects	Females (n=21)
Healthy Participants				
Baseline	12	ref.	8	ref.
Day1	11	1.15* (1.02, 1.30)	7	1.06 (0.92, 1.23)
Day2	12	1.13* (1.01, 1.27)	8	1.16* (1.02, 1.34)
Day3	11	1.21* (1.07, 1.36)	7	1.08 (0.94, 1.25)
Patients with PTSD				
Baseline	3	ref.	7	ref.
Day1	3	1.05 (0.87, 1.27)	4	0.97 (0.82, 1.15)
Day2	3	1.08 (0.90, 1.29)	6	1.10 (0.95, 1.28)
Day3	3	1.15 (0.96, 1.39)	5	1.03 (0.88, 1.21)

[a:] Models controlling for age, BMI, race and education level

*Significantly increase over time ($p<0.05$)

Note: No significant interaction between sex or PTSD diagnosis and Time

Table 5. Ratio of PACAP concentration to the baseline PACAP concentration change over time, grouped by sex and treatment groups, measured from baseline to the end of Day 3.

Table 5. Ratio of PACAP Concentration to Baseline Over Time, Measures from Baseline to Day 3[a] (by sex & VNS Treatment)

(Adjusted β Estimate (95% Confidence Interval))

	No. of Subjects	Sham Stimulation (n=16)	No. of Subjects	VNS Treatment (n=20)
Males				
Baseline	7	ref.	8	ref.
Day1	7	1.14 (0.99, 1.32)	7	1.11 (0.97, 1.28)
Day2	7	1.15* (1.00, 1.33)	8	1.10 (0.96, 1.25)
Day3	7	1.24* (1.08, 1.43)	7	1.15* (1.00, 1.33)
Females				
Baseline	5	ref.	10	ref.
Day1	3	1.04 (0.88, 1.24)	8	1.01 (0.89, 1.16)
Day2	5	1.17* (1.01, 1.37)	9	1.11 (0.98, 1.27)
Day3	4	1.11 (0.94, 1.31)	8	1.03 (0.90, 1.18)

[a:] Models controlling for age, BMI, race and education level
*Significantly increase over time ($p<0.05$)
Note: No significant interaction between sex or treatment effect and Time

Table 6. The Biomarker Statistics by PTSD Diagnosis and Treatment Groups

Table 6. Biomarker statistics between 4 groups

Variables		PTSD						Non-PTSD						Total		
		VNS		Sham		Total		VNS		Sham		Total				Normality Test*
		# of Samples	Blood Levels (Mean ± SD)	# of Samples	Blood Levels (Mean ± SD)	# of Samples	Blood Levels (Mean ± SD)	# of Samples	Blood Levels (Mean ± SD)	# of Samples	Blood Levels (Mean ± SD)	# of Samples	Blood Levels (Mean ± SD)	# of Samples	Blood Levels (Mean ± SD)	
Baselines (pg/ml)	PACAP	5	84.34 ± 45.99	5	92.5 ± 40.13	10	88.42 ± 40.92	13	75.71 ± 38.57	7	91.09 ± 36.44	20	81.09 ± 37.62	30	83.53 ± 38.2	<0.0001
	BDNF	5	255.13 ± 453.13	5	358.51 ± 317.28	10	306.82 ± 372.78	11	281.28 ± 266.7	8	214.53 ± 349.06	19	253.18 ± 296.72	29	271.67 ± 319.28	<0.0001
	IFN-γ	5	11.02 ± 20.29	5	1.96 ± 1.25	10	6.49 ± 14.37	11	2.16 ± 1.2	8	2.92 ± 1.92	19	2.48 ± 1.54	29	3.86 ± 8.46	<0.0001
	IL1β	5	0.06 ± 0.04	5	0.1 ± 0.08	10	0.08 ± 0.06	11	0.05 ± 0.03	8	0.08 ± 0.05	19	0.06 ± 0.04	29	0.07 ± 0.05	0.003
	IL2	5	0.17 ± 0.19	5	0.28 ± 0.21	10	0.23 ± 0.19	11	0.34 ± 0.22	8	0.32 ± 0.21	19	0.33 ± 0.21	29	0.29 ± 0.21	<0.0001
	IL6	5	0.75 ± 0.44	5	0.89 ± 0.49	10	0.82 ± 0.45	11	0.72 ± 0.61	8	0.55 ± 0.31	19	0.65 ± 0.5	29	0.71 ± 0.48	<0.0001
	TNF-α	5	2.6 ± 0.85	5	2.64 ± 1.35	10	2.62 ± 1.06	11	2.9 ± 1.47	8	2.2 ± 0.53	19	2.61 ± 1.2	29	2.61 ± 1.13	<0.0001
	Ghrelin	4	720.66 ± 327.93	4	251.11 ± 186.9	8	485.89 ± 352.21	10	367.47 ± 309.9	7	581.74 ± 353.51	17	455.7 ± 335.71	25	465.36 ± 333.95	0.005
	IL12p70	4	1.65 ± 1.3	4	4.21 ± 0.54	8	2.93 ± 1.65	10	2.69 ± 1.83	7	3.89 ± 1.13	17	3.19 ± 1.65	25	3.1 ± 1.62	0.711
	IL13	4	29.02 ± 19.66	4	52.59 ± 9.31	8	40.8 ± 19.02	10	39.7 ± 17.63	7	54.82 ± 16.97	17	45.93 ± 18.48	25	44.29 ± 18.42	0.979
	IL22	4	1.47 ± 0.78	4	2.64 ± 1.1	8	2.05 ± 1.08	10	2.32 ± 1.22	7	2.7 ± 1.05	17	2.47 ± 1.14	25	2.34 ± 1.11	0.631
	IL5	4	2 ± 1.15	4	2.3 ± 0.91	8	2.15 ± 0.98	10	2.26 ± 1.13	7	2.08 ± 0.95	17	2.19 ± 1.03	25	2.17 ± 0.99	0.317
	MIF	4	21973.54 ± 7972.59	4	10174.35 ± 10222.84	8	16073.94 ± 10573.87	10	24619.33 ± 4311.48	7	18806.99 ± 12313.17	17	22226.02 ± 8718.13	25	20257.35 ± 9584.35	0.015
All Timepoints (pg/ml)	PACAP	15	90.03 ± 41.4	19	96.58 ± 41.01	34	93.69 ± 40.69	50	81.51 ± 37.29	26	102.39 ± 36.12	76	88.65 ± 37.99	110	90.21 ± 38.72	<0.0001
	BDNF	48	180.08 ± 229.47	51	211.88 ± 253.6	99	196.46 ± 241.5	114	274.48 ± 368.13	93	278.46 ± 381.32	207	276.27 ± 373.2	306	250.45 ± 337.95	<0.0001
	IFN-γ	48	9.61 ± 14.93	51	16.51 ± 33.71	99	13.17 ± 26.44	114	2.29 ± 1.29	93	2.75 ± 1.73	207	2.5 ± 1.52	306	5.95 ± 15.85	<0.0001
	IL1β	48	0.1 ± 0.08	51	0.08 ± 0.05	99	0.09 ± 0.07	114	0.08 ± 0.07	93	0.09 ± 0.05	207	0.09 ± 0.06	306	0.09 ± 0.06	<0.0001
	IL2	48	0.26 ± 0.18	51	0.27 ± 0.17	99	0.26 ± 0.18	114	0.31 ± 0.21	93	0.24 ± 0.2	207	0.28 ± 0.21	306	0.27 ± 0.2	<0.0001
	IL6	48	1.13 ± 0.91	51	1.32 ± 1.25	99	1.23 ± 1.1	114	0.88 ± 0.68	93	0.68 ± 0.35	207	0.79 ± 0.56	306	0.93 ± 0.8	<0.0001
	TNF-α	48	2.4 ± 0.7	51	2.6 ± 1.33	99	2.51 ± 1.07	114	2.88 ± 1.28	93	2.44 ± 1.3	207	2.68 ± 1.3	306	2.63 ± 1.23	<0.0001
	Ghrelin	21	598.76 ± 216.68	23	295.9 ± 149.71	44	440.44 ± 238.16	58	352.54 ± 296.76	50	538 ± 361.24	108	438.4 ± 339.56	152	438.99 ± 312.82	0.021
	IL12p70	21	2.35 ± 1.58	23	3.8 ± 1.56	44	3.11 ± 1.72	58	3.18 ± 1.69	50	4.06 ± 1.77	108	3.58 ± 1.77	152	3.45 ± 1.76	<0.0001
	IL13	21	36.54 ± 19.16	23	45.09 ± 15.34	44	41.01 ± 17.6	58	44.41 ± 16.29	50	55.77 ± 27.09	108	49.67 ± 22.58	152	47.16 ± 21.56	<0.0001
	IL22	21	1.81 ± 1.09	23	2.39 ± 1.16	44	2.12 ± 1.15	58	2.61 ± 1.2	50	3.93 ± 4.29	108	3.22 ± 3.1	152	2.9 ± 2.73	0.004
	IL5	21	2.17 ± 1.06	23	2.01 ± 0.73	44	2.08 ± 0.9	58	2.48 ± 1.11	50	2.32 ± 1.02	108	2.41 ± 1.06	152	2.31 ± 1.03	<0.0001
	MIF	21	21531.43 ± 6598.65	23	12852.98 ± 9342.14	44	16994.97 ± 9172.34	58	22996.69 ± 6052.21	50	18779.77 ± 10486.61	108	21044.41 ± 8621.77	152	19872.21 ± 8945.81	<0.0001

*: P-value is provided by Shapiro-Wilk test for normality. $P < 0.05$ is considered as a violation of the normality.

Table 7. Correlation of PACAP and Cytokines Concentration in Peripheral Blood (Over All Time Points)

Table 7. Correlation of PACAP and cytokines in PB

Variables (Log-transformed)	β Estimate[a]	Standard Deviation	P-values
BDNF	-0.03	0.03	0.3524
IFN-γ*	0.14	0.06	0.0215
IL1β	-0.08	0.05	0.1115
IL2	0.04	0.05	0.3715
IL6*	-0.13	0.06	0.0476
TNF-α	-0.17	0.14	0.2182
Ghrelin*	0.15	0.08	0.0499
IL12p70	-0.15	0.08	0.0517
IL13*	-0.25	0.09	0.0060
IL22	-0.16	0.08	0.0579
IL5*	-0.27	0.11	0.0132
MIF	0.03	0.04	0.4722

*: Significantly associated with PACAP blood levels
a: Model adjusted for age, BMI, sex, race and education level

FIGURES & FIGURE LEGENDS

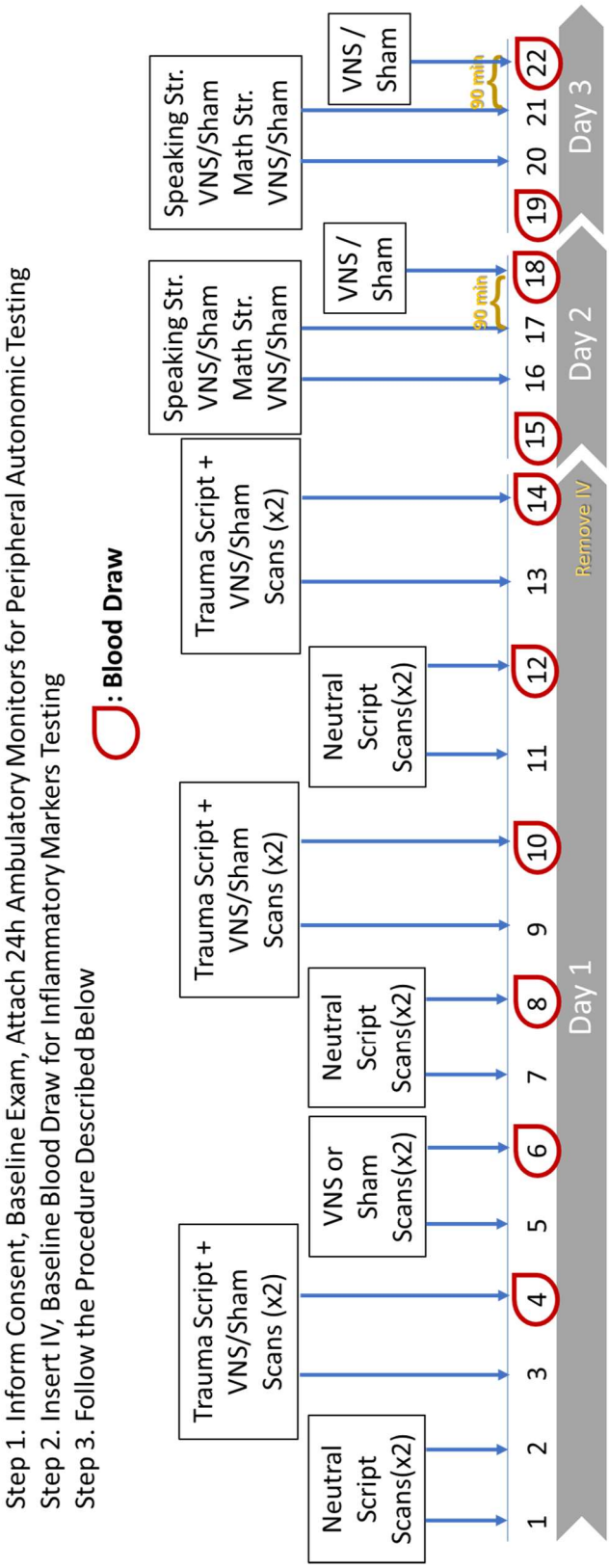


Figure 1. Timeline depiction of the study assessing the potency and kinetics of VNS in the context of stressful scripts.

Figure 1. Timeline depiction of the study assessing the potency and kinetics of VNS treatment in the context of stressful scripts, speech task and math challenge. Blood was drawn at the baseline and the circled time points which immediately after the scans or VNS treatment / Sham stimulation in Day 1. In Day 2 and Day 3, blood was drawn at 90 minutes after the VNS treatment / Sham stimulation

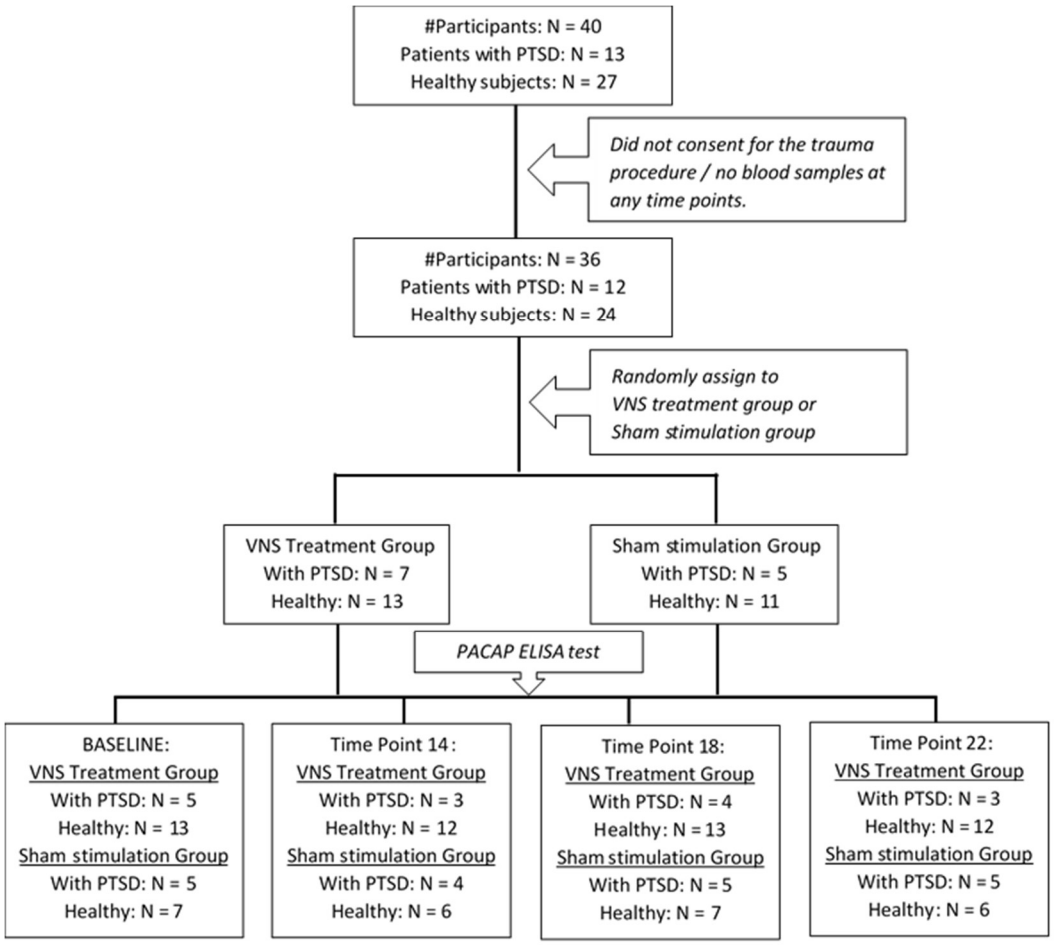


Figure 2. The flow diagram of subjects' eligibility and numbers including in the following analysis.

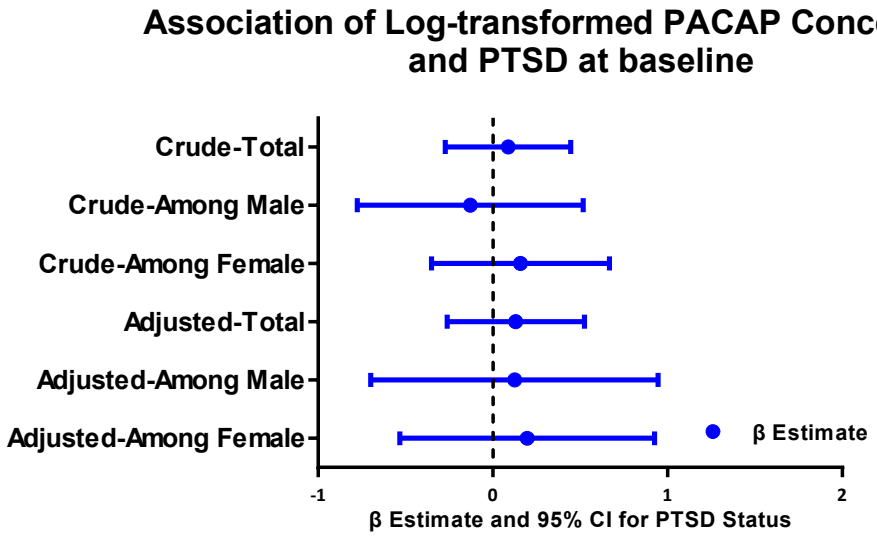


Figure 3. The association of log-transformed PACAP concentration with PTSD status at the baseline. Measured both in crude and adjusted for age, sex BMI, race and education, grouped by Sex. Coded PTSD diagnosis as 1, healthy as 0

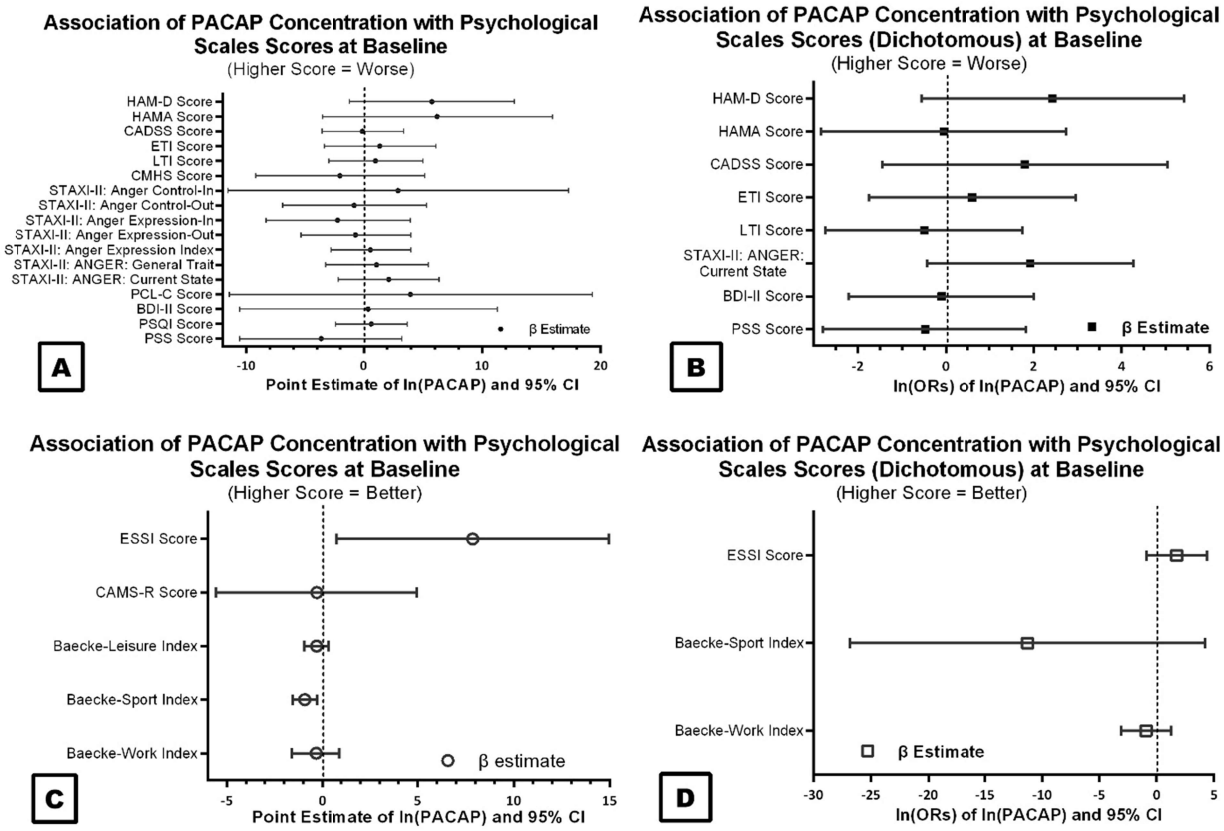
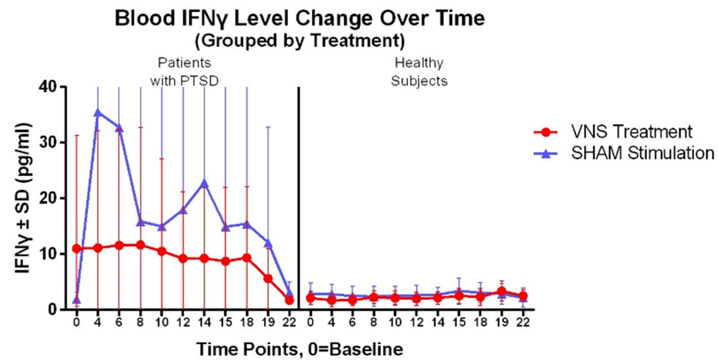
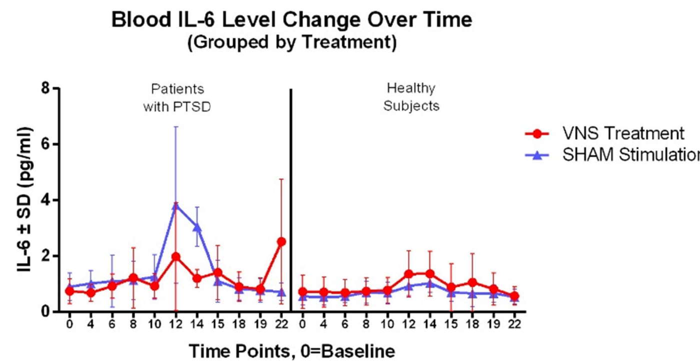


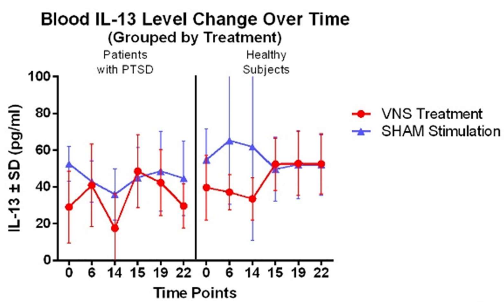
Figure 4. The association of log-transformed PACAP concentration with scores of psychological and functional scales at the baseline. Models adjusted for age, sex, BMI, race and education. Higher scores of the scales listed in Figure 4A and 4B indicated a worse physical or psychological condition, which indicated a better condition for scales in 4C and 4D. Measuring the association between log-transformed PACAP concentration with dichotomous scores if the score did not follow a normal distribution (4B, 4D).



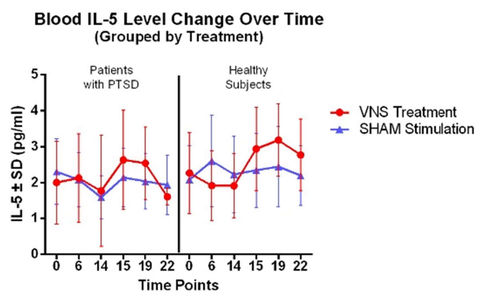
Sample	Patients with PTSD											Healthy Subjects										
Number:	0	4	6	8	10	12	14	15	18	19	22	0	4	6	8	10	12	14	15	18	19	22
VNS	5	5	5	5	5	4	4	5	4	4	2	11	10	10	11	11	10	11	11	8	11	10
Sham	5	5	4	5	5	4	3	5	5	5	5	8	8	9	9	9	9	7	9	7	9	9



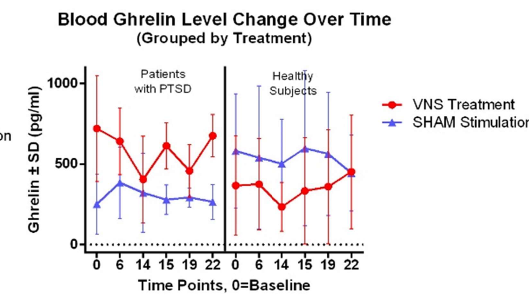
Sample	Patients with PTSD											Healthy Subjects										
Number:	0	4	6	8	10	12	14	15	18	19	22	0	4	6	8	10	12	14	15	18	19	22
VNS	5	5	5	5	5	4	4	5	4	4	2	11	10	10	11	11	10	11	11	8	11	10
Sham	5	5	4	5	5	4	3	5	5	5	5	8	8	9	9	9	9	7	9	7	9	9



Sample	Patients with PTSD						Healthy Subjects					
Number:	0	6	14	15	19	22	0	6	14	15	19	22
VNS	4	4	2	4	4	3	10	10	10	9	10	9
Sham	4	3	4	4	4	4	7	9	7	9	9	9



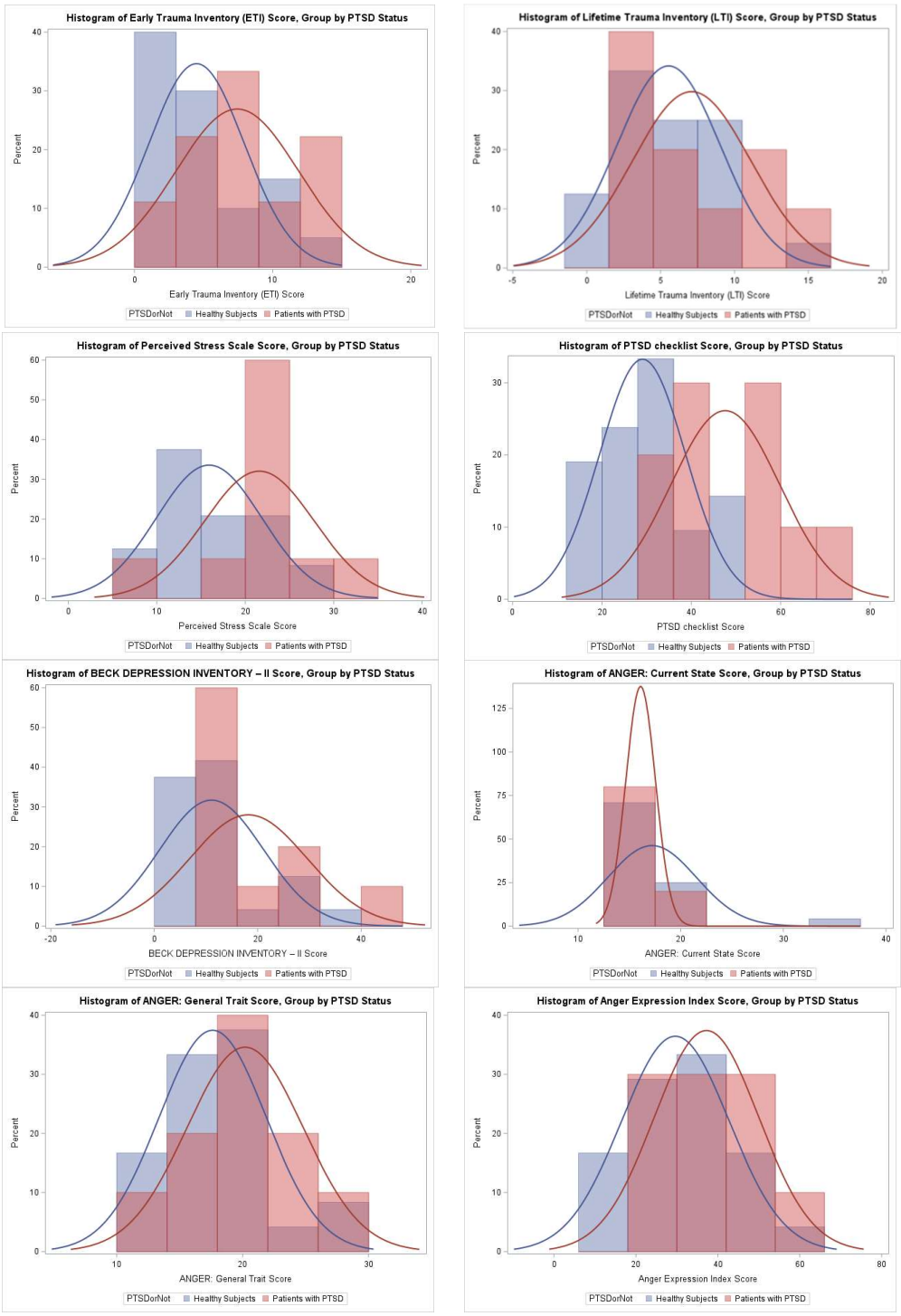
Sample	Patients with PTSD						Healthy Subjects					
Number:	0	6	14	15	19	22	0	6	14	15	19	22
VNS	4	4	2	4	4	3	10	10	10	9	10	9
Sham	4	3	4	4	4	4	7	9	7	9	9	9



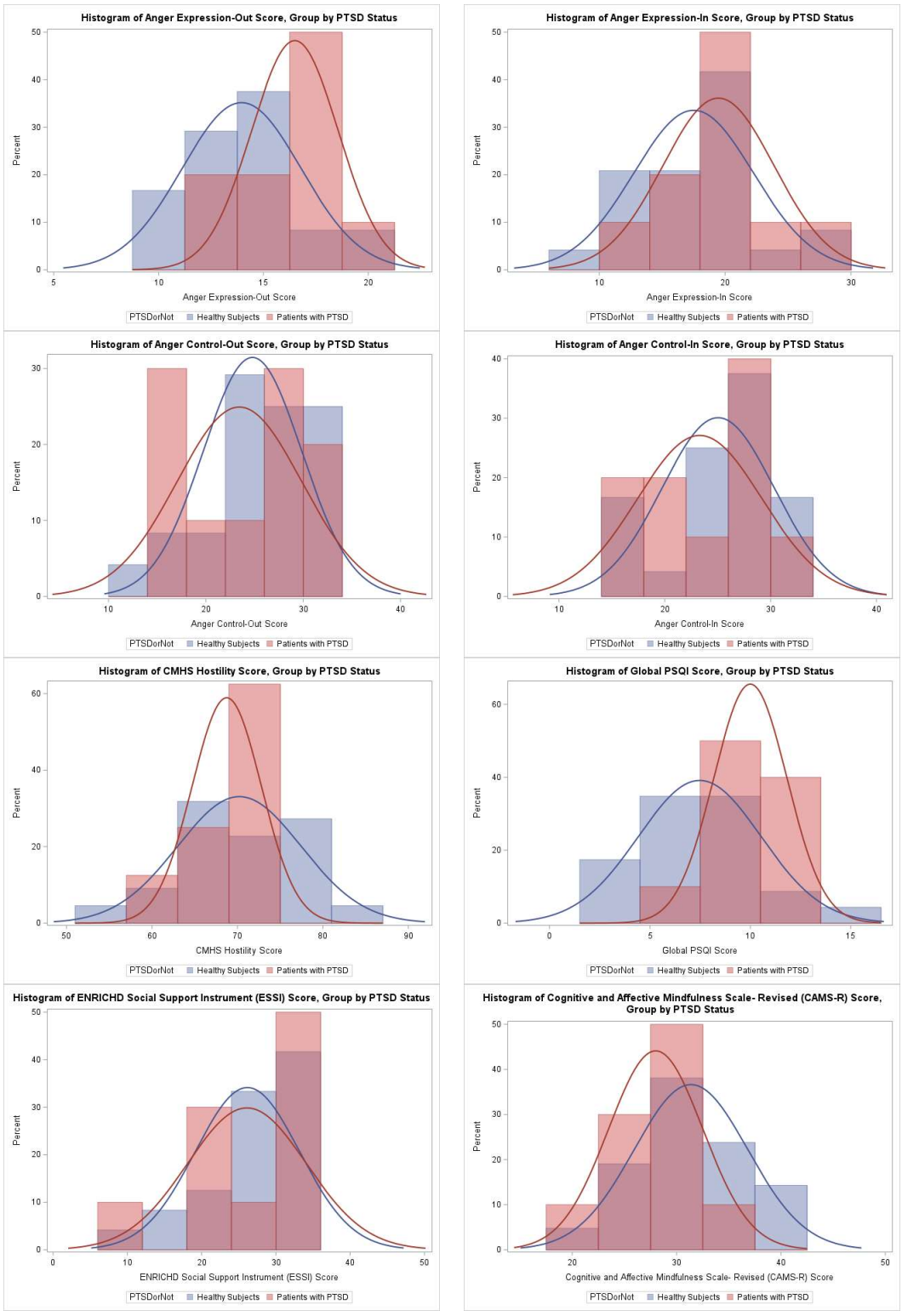
Sample	Patients with PTSD						Healthy Subjects					
Number:	0	6	14	15	19	22	0	6	14	15	19	22
VNS	4	4	2	4	4	3	10	10	10	9	10	9
Sham	4	3	4	4	4	4	7	9	7	9	9	9

Figure 5. The average blood levels of IFN-γ, IL-6, IL-13, IL-5 and Ghrelin at each time points (mean with standard deviation). Biomarker concentration was grouped by PTSD diagnosis and intervention method (VNS treatment or Sham stimulation). Time point 15 = Day 2 baseline, Time point 19 = Day 3 baseline. The number of subjects measured in each time points were listed below each figure.

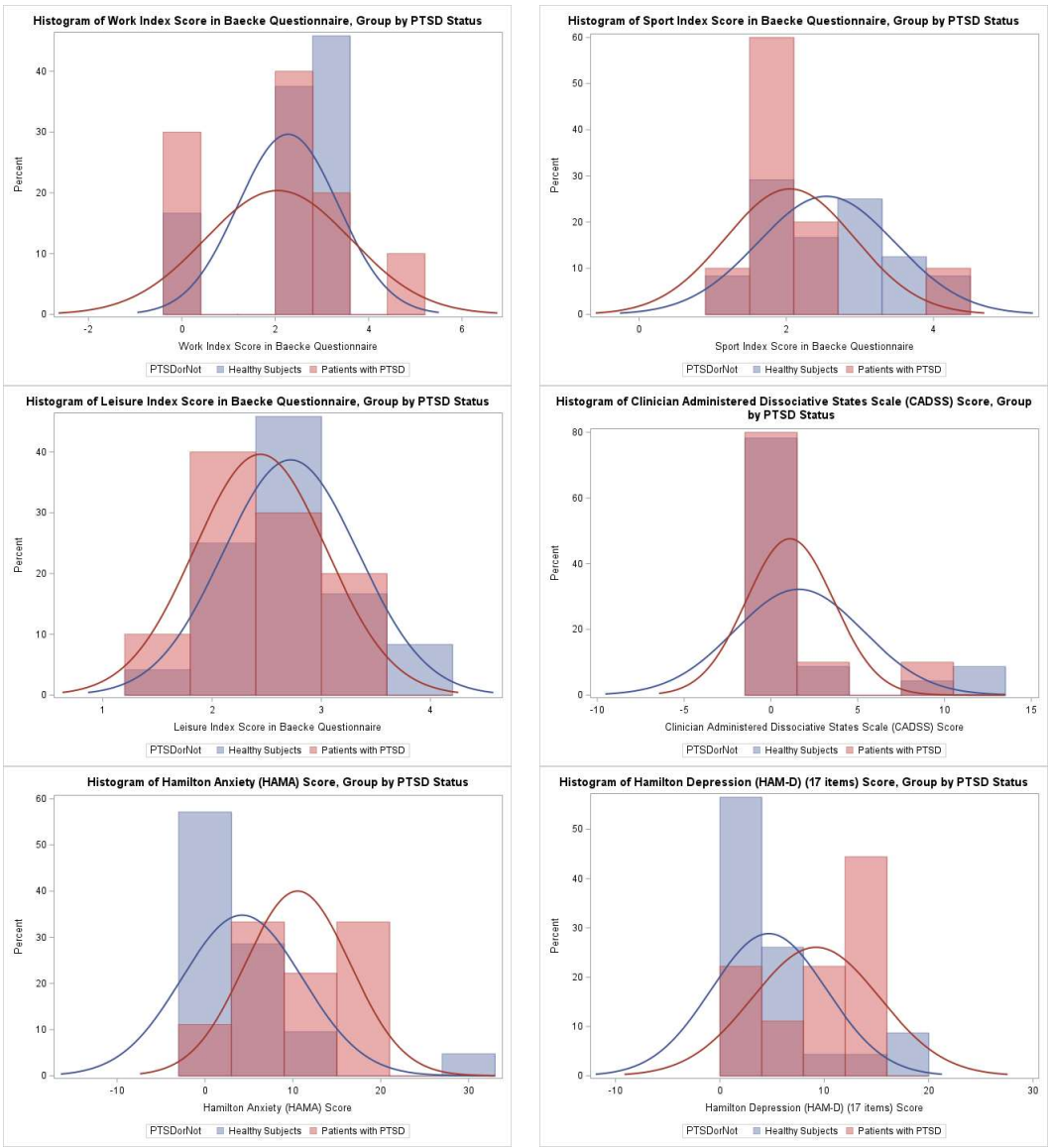
APPENDICES



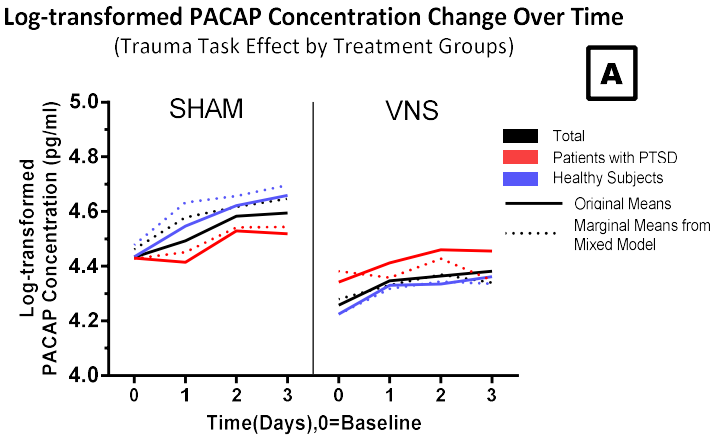
Appendix Figure 1. The histograms of all psychological scores. (Part 1: Early Trauma Inventory (ETI); Lifetime Trauma Inventory (LTI); Perceived Stress Scale (PSS); PTSD Checklist (PCL-C); BECK DEPRESSION INVENTORY – II; State-Trait Anger Expression Inventory (STAXI-II) subscales: Current State, General Trait, Expression Index)



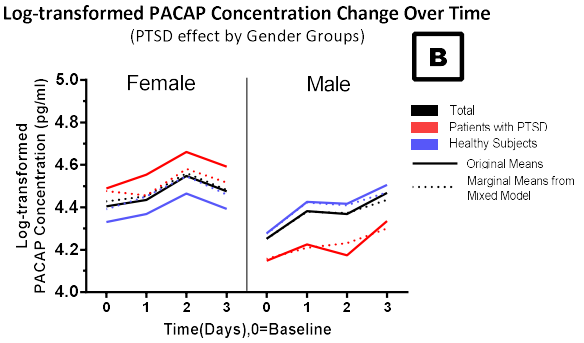
Appendix Figure 1. The histograms of all psychological scores. (Part 2: State-Trait Anger Expression Inventory (STAXI-II) subscales: Expression-Out, Expression-In, Control-Out, Control-In; Cook-Medley Hostility Scale (CMHS); Pittsburgh Sleep Quality Index (PSQI); ENRICHD Social Support Instrument (ESSI); Cognitive and Affective Mindfulness Scale (CAMS-R))



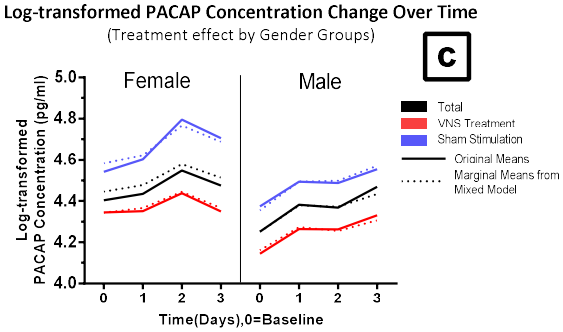
Appendix Figure 1. The histograms of all psychological scores. (Part 3: Baecke Activity Questionnaire subscales: Work Index, Sport Index, Leisure Index; Clinician Administered Dissociative States Scale (CADSS); Hamilton Anxiety (HAMA); Hamilton Depression (HAM-D) (17 items))



	Healthy Subjects		Patients with PTSD		Total	
	Original	Predicted	Original	Predicted	Original	Predicted
Sham Stimulation						
Baseline	4.43 ± 0.44	4.48 ± 0.3	4.43 ± 0.53	4.43 ± 0.46	4.43 ± 0.45	4.46 ± 0.34
Day 1	4.55 ± 0.45	4.63 ± 0.28	4.41 ± 0.51	4.45 ± 0.42	4.49 ± 0.45	4.58 ± 0.34
Day 2	4.62 ± 0.34	4.66 ± 0.31	4.53 ± 0.57	4.54 ± 0.43	4.58 ± 0.43	4.62 ± 0.35
Day 3	4.66 ± 0.36	4.7 ± 0.29	4.52 ± 0.38	4.54 ± 0.43	4.59 ± 0.36	4.65 ± 0.35
VNS Treatment						
Baseline	4.23 ± 0.45	4.22 ± 0.39	4.34 ± 0.45	4.38 ± 0.34	4.26 ± 0.44	4.28 ± 0.37
Day 1	4.33 ± 0.47	4.32 ± 0.39	4.41 ± 0.52	4.36 ± 0.33	4.35 ± 0.46	4.33 ± 0.37
Day 2	4.33 ± 0.33	4.34 ± 0.39	4.46 ± 0.49	4.43 ± 0.36	4.36 ± 0.36	4.37 ± 0.38
Day 3	4.36 ± 0.43	4.34 ± 0.39	4.46 ± 0.48	4.35 ± 0.37	4.38 ± 0.43	4.34 ± 0.38



	Healthy Subjects		Patients with PTSD		Total	
	Original	Predicted	Original	Predicted	Original	Predicted
Male Subjects						
Baseline	4.28 ± 0.45	4.28 ± 0.38	4.15 ± 0.51	4.16 ± 0.39	4.25 ± 0.45	4.25 ± 0.37
Day 1	4.42 ± 0.43	4.42 ± 0.37	4.22 ± 0.42	4.21 ± 0.32	4.38 ± 0.42	4.38 ± 0.37
Day 2	4.42 ± 0.38	4.41 ± 0.38	4.17 ± 0.42	4.23 ± 0.35	4.37 ± 0.38	4.37 ± 0.38
Day 3	4.5 ± 0.38	4.47 ± 0.38	4.33 ± 0.3	4.3 ± 0.35	4.47 ± 0.36	4.43 ± 0.38
Female Subjects						
Baseline	4.33 ± 0.46	4.39 ± 0.38	4.49 ± 0.44	4.48 ± 0.36	4.4 ± 0.45	4.43 ± 0.36
Day 1	4.37 ± 0.55	4.45 ± 0.36	4.55 ± 0.51	4.46 ± 0.35	4.43 ± 0.52	4.45 ± 0.36
Day 2	4.46 ± 0.34	4.55 ± 0.39	4.66 ± 0.49	4.58 ± 0.38	4.55 ± 0.41	4.56 ± 0.38
Day 3	4.39 ± 0.51	4.46 ± 0.37	4.59 ± 0.43	4.52 ± 0.39	4.48 ± 0.47	4.48 ± 0.38



	Sham Stimulation		VNS Treatment		Total	
	Original	Predicted	Original	Predicted	Original	Predicted
Male Subjects						
Baseline	4.37 ± 0.48	4.35 ± 0.43	4.14 ± 0.37	4.16 ± 0.31	4.25 ± 0.45	4.25 ± 0.37
Day 1	4.49 ± 0.45	4.49 ± 0.4	4.26 ± 0.33	4.27 ± 0.31	4.38 ± 0.42	4.38 ± 0.37
Day 2	4.49 ± 0.43	4.5 ± 0.41	4.26 ± 0.3	4.25 ± 0.31	4.37 ± 0.38	4.37 ± 0.38
Day 3	4.55 ± 0.41	4.57 ± 0.41	4.33 ± 0.28	4.31 ± 0.31	4.47 ± 0.36	4.43 ± 0.38
Female Subjects						
Baseline	4.54 ± 0.29	4.58 ± 0.28	4.34 ± 0.43	4.34 ± 0.39	4.4 ± 0.45	4.45 ± 0.36
Day 1	4.6 ± 0.29	4.62 ± 0.27	4.35 ± 0.42	4.37 ± 0.39	4.43 ± 0.52	4.48 ± 0.36
Day 2	4.79 ± 0.31	4.77 ± 0.29	4.44 ± 0.43	4.44 ± 0.41	4.55 ± 0.41	4.58 ± 0.4
Day 3	4.7 ± 0.29	4.69 ± 0.28	4.35 ± 0.42	4.37 ± 0.42	4.48 ± 0.47	4.51 ± 0.39

Appendix Figure 2. The means and marginal means of log-transformed PACAP concentration change over time, grouped by treatment group (Appendix Figure 2A) or sex group (Appendix Figure 2B & 2C). Solid lines were plotted with the actual data, and the dotted lines were plotted using the marginal means from the mixed model, which adjusted for age, BMI, sex (if did not stratify by sex), race and education levels, and considering the heterogeneity across individuals and time change. In appendix figure 2A & 2B, the red lines were drawn with the data of patients with PTSD, blue lines were drawn with the data of healthy subjects, and black lines were drawn with all subjects' data. In appendix figure 2C, the red lines were drawn with the data of VNS treatment, blue lines were drawn with the data of sham stimulation, and black lines were drawn with all subjects' data.