



Global arginine bioavailability, a marker of nitric oxide synthetic capacity, is decreased in PTSD and correlated with symptom severity and markers of inflammation



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ABSTRACT

Introduction: Psychiatric, physical and biological aspects of posttraumatic stress disorder (PTSD) may be associated with dysfunctions in several cellular processes including nitric oxide (NO) production. NO is synthesized from arginine in a reaction carried out by NO synthase (NOS) enzymes. The recently introduced “global arginine bioavailability ratio” (GABR; ratio of arginine to [ornithine + citrulline]) has been proposed as a reliable approximation of NO synthetic capacity in vivo. The objectives of the present study were to test the hypotheses that (i) subjects with combat-related PTSD have lower GABR scores than combat controls, (ii) GABR score is inversely associated with the severity of psychopathological measures, (iii) GABR score is inversely associated with markers of inflammation.

Methods: Metabolic profiling for plasma samples (i.e. arginine, citrulline and ornithine) and inflammation markers (interleukin [IL]-6, IL-1 β , tumor necrosis factor [TNF]- α , interferon [IFN]- γ and C-reactive protein [CRP]) were assessed in 56 combat-exposed males with PTSD and 65 combat-exposed males without PTSD. We assessed severity of PTSD (Clinician Administered PTSD Scale [CAPS]) and depression (Beck Depression Inventory-II [BDI-II]) as well as history of early life trauma (Early Trauma Inventory [ETI]) and affectivity (Positive and Negative Affect Schedule [PANAS]).

Results: The GABR value was (i) significantly lower in PTSD subjects compared to controls ($p = 0.001$), (ii) significantly inversely correlated with markers of inflammation including IL6 ($p = 0.04$) and TNF α ($p = 0.02$), and (iii) significantly inversely correlated with CAPS current ($p = 0.001$) and lifetime ($p < 0.001$) subscales, ETI ($p = 0.045$) and PANAS negative ($p = 0.006$). Adding antidepressant use or MDD diagnosis as covariates led to similar results. Adding age and BMI as covariates also led to similar results, with the exception of IL6 and ETI losing their significant association with GABR.

Discussion: This study provides the first evidence that global arginine bioavailability, a marker of NO synthetic capacity in vivo, is lower in veterans with PTSD and is negatively associated with some markers of inflammation as well as with measures of PTSD symptom severity, negative affectivity and childhood adverse experiences. These findings add to the accumulating evidence that specific cellular dysfunction may be associated with the symptomatology of PTSD and may help to explain the higher burden of cardio-metabolic disturbances seen in this disorder.

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1. Introduction

Post-traumatic stress disorder (PTSD) is a debilitating mental illness characterized by recurrent distressing memories of an initial traumatic event, emotional numbing and hyperarousal (American Psychiatric Association, 2013). In addition to the traditional psychiatric symptoms, individuals with PTSD have a substantially higher medical burden, with increased rates of cardiometabolic disturbances and early mortality, suggesting widespread physical and biological concomitants of the disease (Levine et al., 2014).

Recent evidence suggests that the psychiatric, physical and biological aspects of PTSD and related stress disorders may be associated with significant dysfunctions in several cellular processes including alterations in inflammation (Lindqvist et al., 2014; Wilson et al., 2013), oxidative stress (Cepnija et al., 2011; Wilson et al., 2013), telomere homeostasis (Jergovic et al., 2014; O'Donovan et al., 2011), neuroendocrine regulation (Rasmussen et al., 2010; Yehuda, 2001), mitochondrial activity (Li et al., 2014; Mellon et al., 2015; Su et al., 2008) and nitric oxide (NO) production (Bugajski, 1999; Harris et al., 2000; Lopez-Figueroa et al., 1998; Persoons et al., 1995; Yeh et al., 2002).

Appropriate levels of endogenous NO play an important role in the protection against the onset and progression of cardiovascular disease (Pacher et al., 2007). The cardioprotective roles of NO include regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and prevention of smooth muscle cell proliferation (Pacher et al., 2007). In addition, NO can influence many aspects of the inflammatory cascade and may play a critical role in the physiopathology of both acute and chronic inflammatory diseases (Laroux et al., 2001). NO may also regulate stress responses, and abnormalities of NO have been posited in clinical or pre-clinical studies on Acute Stress Disorder (ASD) and PTSD (Bugajski, 1999; Harris et al., 2000; Lopez-Figueroa et al., 1998; Persoons et al., 1995; Yeh et al., 2002). For example, Yeh et al. measured NO concentration *in vivo* in a sample of people with ASD finding that (i) male military personnel affected by ASD had lower plasma NO levels than controls, and (ii) there was a significant inverse correlation between the severity of stress-related symptoms and the plasma concentration of NO in ASD subjects (Yeh et al., 2002). Given the role of NO in the modulation of stress response as well as in the etiopathogenesis of stress-related changes involving inflammatory and cardiometabolic systems, we speculate that NO play a significant role in the pathophysiology of PTSD.

NO is exclusively synthesized from arginine in a multistep reaction carried out by NO synthase (NOS) enzymes, producing NO and citrulline (Huynh and Chin-Dusting, 2006). Arginine is also the substrate for arginase, the enzyme that generates urea while converting arginine to ornithine (Huynh and Chin-Dusting, 2006) (Fig. 1).

Despite its clinical relevance, precise measurements of NO have not been performed as part of clinical care mainly due to its very

short physiological half life and the extremely small quantities (picomolar) which are produced (Hlatky et al., 2003). The recently introduced “global arginine bioavailability ratio” (GABR) has been proposed as a reliable approximation of the overall balance of arginine levels and as a comprehensive concept of NO synthetic capacity *in vivo* (Tang et al., 2009, 2013). GABR is calculated by dividing plasma amounts of arginine by plasma amounts citrulline and ornithine {arginine/(citrullin + ornithine)}. The ratio not only accounts for the substrate arginine but also for the metabolic products citrulline and ornithine, which together provide a more meaningful assessment of overall arginine and NO bioavailability than does arginine alone (Tang et al., 2009). Reduced GABR values, indicative of reduced NO synthetic capacity, have been associated with heightened long-term risk for atherosclerosis and major adverse cardiac events (Sourij et al., 2011; Tang et al., 2009, 2013).

Based on the idea that reduced NO synthetic capacity may be associated with some of the PTSD-related psychiatric and somatic manifestations, we hypothesized that patients with PTSD have lower GABR levels than controls. Therefore, the objectives of the present study were to test the following hypotheses: (i) subjects (war veterans) with PTSD have lower GABR scores than psychiatrically healthy combat controls, (ii) GABR score is inversely associated with the severity of psychopathological measures, (iii) GABR score is inversely associated with markers of inflammation (i.e. markers of PTSD-related cellular dysfunction) previously explored by our research team (Lindqvist et al., 2014).

2. Methods

2.1. Ethical statement

The Institutional Review Boards of Icahn School of Medicine at Mount Sinai (ISMMS; New York, NY), the James J. Peters Veterans Administration Medical Center (JJPVAMC; Bronx, New York), New York University Medical Center (NYU; New York, NY), and the University of California, San Francisco, Medical Center (San Francisco, CA) approved this study. Study participants gave written and informed consent to participate. The study was conducted in accordance with the provisions of the Helsinki Declaration.

2.2. Recruitment procedures and study participants

121 Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) male combat veterans participated. Fifty-six of the participants were diagnosed with current PTSD, while 55 did not have PTSD (i.e. controls). Among the PTSD subjects, 28 were diagnosed with concurrent Major Depressive Disorder (MDD). Participants were recruited by NYU and ISMMS/JJPVAMC. Subjects were recruited from the Mental Health Services of the Manhattan, Bronx and Brooklyn Veterans Affairs Medical Centers, other regional VA medical centers, Veterans Service Organizations, National Guard, reservist agencies and organizations and from the general community. Recruitment methods included flyers, in-person presentations, media advertisements, internet postings (e.g. Craigslist) and referral from clinicians. Participants were compensated for their participation. Criteria for inclusion were: (a) PTSD subjects were positive for the presence of current combat-related PTSD of at least 3 months duration, as defined by the DSM-IV (First, 1997), and the Clinician Administered PTSD Scale (CAPS) (Blake et al., 1990) criteria with a current CAPS score >40; (b) control subjects were also combat-exposed but were negative for lifetime PTSD and had a current CAPS score <20; (c) age between 20 and 60; (d) males; and (e) proficient in the English language. The following exclusion criteria were employed for all subjects: (a) history of alcohol dependence within the past 8 months; (b) history

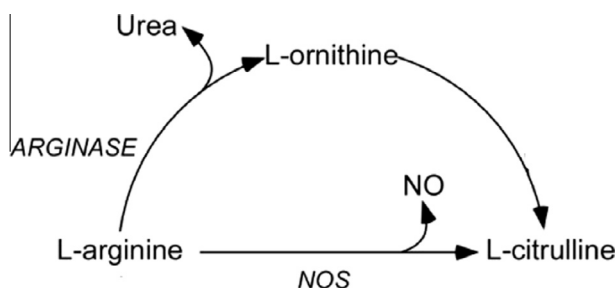


Fig. 1. Schematic illustration of pathways for arginine catabolism. Adapted from (Tang et al., 2009). Abbreviations: NO = nitric oxide; NOS = nitric oxide synthase.

of drug abuse or dependence (except nicotine dependence) within the past year; (c) lifetime history of any psychiatric disorder with psychotic features, bipolar disorder, or obsessive–compulsive disorder; (d) those who were currently exposed to recurrent trauma or have been exposed to a traumatic event within the past 3 months; (e) subjects with prominent suicidal or homicidal ideation; (f) neurologic disorder or systemic illness affecting central nervous system function; (g) history of anemia, recent blood donation in the past 2 months; (i) subjects who were not stable for 2 + months on psychiatric medication, anticonvulsants, antihypertensive medication or sympathomimetic medication; (j) subjects who were classified with a moderate or severe traumatic brain injury (TBI) on the Ohio State University TBI Identification Method-Short Form; and finally (k) subjects who experienced loss of consciousness for greater than 10 min. All study participants, including those who did not have PTSD, experienced combat traumas described in criterion A of DSM-IV PTSD diagnostic criteria. Structured Clinical Interviews for DSM-IV disorders (SCID) (First, 1997) were conducted by doctoral level psychologists, and were audio recorded and calibrated weekly with a senior clinician in the PTSD program. The present sample partially overlaps with that described in Mellon et al. (2015), which was a metabolomic study on more than 350 metabolites, and in Lindqvist et al. (2014), which was a study on inflammatory cytokines, with each of the three studies including subjects with all pertinent data available at the time of manuscript preparation.

2.3. Psychiatric and psychological assessment measures

The SCID was used to determine whether participants met DSM-IV diagnostic criteria for any psychiatric disorder (First, 1997). The CAPS (score range: 0–136; Cronbach's $\alpha = 0.97$) was used to determine the severity of current PTSD symptoms (past month; "CAPS current") and the severity of the most severe lifetime episode of combat-related PTSD ("CAPS lifetime") (Blake et al., 1990; Pupo et al., 2011). The self-rated Beck Depression Inventory-II (BDI-II; score range: 0–63; Cronbach's $\alpha = 0.91$) was used to assess the depression symptom severity (Beck et al., 1996). The Early Trauma Inventory (ETI)–Self Report Short Form (score range: 0–27; Cronbach's $\alpha = 0.95$) was used to assess the exposure to early life trauma (Bremner et al., 2007). The positive affect and negative affect subscales (score ranges: 10–50; Cronbach's $\alpha > 0.84$) of the Positive and Negative Affect Schedule (PANAS) were used to assess positive and negative affectivity (Watson et al., 1988).

2.4. Blood sampling and cytokine assays

Blood was drawn in the morning after a night of fasting. Whole blood was collected into 10 ml SST tubes (Becton Dickinson, Franklin Lakes, NJ). Serum was allowed to clot for 30 min at room temperature, then spun (1300 rpm for 15 min), frozen, and stored at -80°C for subsequent cytokine quantification. A high sensitivity multiplexed sandwich immunoassay was used to quantify interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ concentrations (Mesoscale Discovery, Gaithersburg, MD). C-reactive protein (CRP) was assayed with a latex-enhanced immunoturbidimetric method (Sonora Quest Laboratories). More details about assay methodology can be found in our previously published study (Lindqvist et al., 2014).

2.5. Profiling of arginine, ornithine and citrulline

Metabolic profiling for plasma samples was performed at Metabolon, Inc. (Durham, NC) using three independent platforms: ultra-high performance liquid chromatography/tandem mass

spectrometry (UHPLC/MS/MS2) optimized for basic species, UHPLC/MS/MS2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were processed as described (Evans et al., 2009; Ohta et al., 2009). For each sample, 100 μL of plasma was used for analyses. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, UT), protein was precipitated from the plasma with methanol that contained four standards, used to determine extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Aliquots, dried under nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50 μL 0.1% formic acid in water (acidic conditions) or in 50 μL 6.5 mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHPLC/MS/MS2 analyses, or were derivatized at 60 $^{\circ}\text{C}$ for one hour using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture a cetoneitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine, to a final volume of 50 μL for GC/MS analysis. In addition, three types of controls were analyzed in concert with the experimental samples: aliquots of a well-characterized human plasma pool served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed monitoring of instrument performance. Experimental samples and controls were randomized across platform run days. For UHPLC/MS/MS2 analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA), which consisted of an electrospray ionization source and linear ion-trap mass analyzer. The MS instrument scanned 99–1000 m/z and alternated between MS and MS2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60 $^{\circ}\text{C}$ to 340 $^{\circ}\text{C}$ and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50–750 atomic mass unit scan range. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, in-source fragments, and associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon (Dehaven et al., 2010). Instrument variability was determined by calculating the median relative standard deviation for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median relative standard deviation for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the matrix samples, which are technical replicates of pooled samples. Values for instrument and process variability were 6% and 12%, respectively.

2.6. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) was used for statistical calculations. All tests were 2-tailed with an $\alpha = 0.05$. Significance values between 0.05 and 1.00 are reported as trends. Data are expressed as means \pm SD. In order to minimize the risk of Type I statistical error due to multiple comparisons, we summarized the values of metabolites (i.e. arginine, ornithine and citrulline) into a single GABR score (described above), and the values of cytokines (i.e. IL6, IL1 β , TNF α , IFN γ and CRP) into a single "total pro-inflammatory" score (by adding standardized z-scores of each cytokine) (Lindqvist et al., 2014). Total pro-inflammatory, GABR, ETI, BDI-II and PANAS negative scores were non-normally distributed thus they were transformed into

normality using the Blom transformation, a statistical procedure replacing each raw score with its rank value and adjusting the scale distances between the ranks to achieve a normal distribution (Blom, 1958). Mann–Whitney *U*-test for continuous variables or a chi-square test for dichotomous variables were used to examine participants' baseline between-group differences. One-way analysis of variance (ANOVA) was used to test for GABR group differences between veterans with or without PTSD and, Pearson correlations were used to determine the association of GABR with clinical assessments and the total pro-inflammatory score. In our sample we found a significant inverse correlations between BMI and GABR ($r = -0.25$; $p = 0.007$). In addition, prior literature has suggested that NO production may be inversely correlated with age (Goubareva et al., 2007); therefore, we performed an extended model using age and BMI as covariates in both intergroup comparison (ANCOVAs) and correlation analyses. ANOVAs with Bonferroni post-hoc analyses and ANCOVAs were also performed to determine the possible contribution of MDD diagnosis and antidepressant use to the inter-group (PTSD vs controls) differences in GABR values.

3. Results

3.1. Baseline characteristics of the sample

Demographic and clinical characteristics of the subjects are presented in Table 1. There were no significant differences between groups by age, years of education or smoking status. Hispanic ethnicity was significantly more frequently represented in the PTSD sample. Subjects with PTSD had a significantly higher average BMI than controls ($p = 0.026$). Consistent with the recommended treatments for PTSD (Albucher and Liberzon, 2002) and the higher proportion of PTSD subjects having comorbid diagnoses of MDD ($p < 0.001$), the use of antidepressant drugs was more common in the PTSD group ($p < 0.001$). However, the PTSD and combat control groups did not significantly differ in their use of other kinds of drugs (i.e. statins, non-steroidal anti-inflammatory drugs (NSAIDs), anti-diabetic drugs, analgesics, hormones, antibiotics). Subjects with concomitant somatic disorders (clinical hypertension, stable angina, prostate cancer, heart arrhythmias, asthma/allergies and diabetes) were equally distributed across groups. Subjects with

Table 1
Demographic and clinical characteristics of subjects with PTSD and controls.

	PTSD N: 56	Controls N: 65	Mann–Whitney <i>U</i> -test	χ^2
Age (years, mean \pm SD)	33.91 \pm 8.43	32.81 \pm 8.40	0.329	
Years of education (mean \pm SD)	12.86 \pm 3.90	13.20 \pm 5.39	0.120	
Gender	All males	All males		
Smokers (<i>n</i>)	9	4		0.136
BMI (mean \pm SD)	30.12 \pm 5.26	28.07 \pm 4.65	0.026*	
Hispanic/non-hispanic (<i>n</i>)	29/27	21/44		0.029*
<i>Cytokines and metabolites</i>				
TNF alfa (pg/mL, mean \pm SD)	4.28 \pm 4.03	3.07 \pm 0.71	0.043*	
IL1 β (pg/mL, mean \pm SD)	0.15 \pm 0.17	0.11 \pm 0.10	0.466	
IL 6 (pg/mL, mean \pm SD)	11.38 \pm 73.42	0.82 \pm 0.79	0.001*	
IFN γ (pg/mL, mean \pm SD)	1.44 \pm 1.82	1.21 \pm 3.90	0.085	
CRP (mean \pm SD)	3.23 \pm 5.46	1.57 \pm 2.25	0.016*	
Total pro-inflammatory score (mean \pm SD)	0.28 \pm 1.03	-0.26 \pm 0.88	0.007*	
GABR (raw, mean \pm SD)	0.46 \pm 0.17	0.57 \pm 0.19	0.001 ^{ab}	
Ornithine (raw, mean \pm SD) ^a	1.24 \pm 0.52	1.05 \pm 0.43	0.029*	
Citrulline (raw, mean \pm SD) ^a	0.99 \pm 0.23	1.02 \pm 0.21	0.529	
Arginine (raw, mean \pm SD) ^a	0.99 \pm 0.36	1.13 \pm 0.29	0.009*	
<i>Medications</i>				
Taking statins (<i>n</i>)	2	1		0.558
Taking antidepressants (<i>n</i>)	17	2		<0.001*
Taking NSAIDs (<i>n</i>)	4	2		0.364
Taking antidiabetic drugs	1	1		1.000
Taking analgesics	1	0		0.315
Taking hormonal drugs for prostate cancer (<i>n</i>)	0	1		0.315
Taking antibiotics (<i>n</i>)	0	1		0.315
<i>Comorbid diseases</i>				
Clinical hypertension (<i>n</i>)	9	6		0.368
Stable angina (<i>n</i>)	3	1		0.262
Heart arrhythmias (<i>n</i>)	1	0		0.298
Diabetes (<i>n</i>)	3	1		0.326
Prostate cancer (<i>n</i>)	0	1		0.425
Asthma/allergies	2	4		0.68
<i>Clinical measures</i>				
CAPS total current (mean \pm SD)	68.95 \pm 16.77	3.23 \pm 5.17	<0.001*	
CAPS total lifetime (mean \pm SD)	92.25 \pm 15.62	8.77 \pm 8.75	<0.001*	
ETI total score (mean \pm SD) ^a	7.20 \pm 5.69	5.09 \pm 4.34	0.054	
BDI-II (mean \pm SD)	13.36 \pm 3.34	6.28 \pm 4.03	<0.001*	
PANAS positive	24.96 \pm 7.37	34.08 \pm 9.27	<0.001*	
PANAS negative	28.26 \pm 9.35	15.22 \pm 5.01	<0.001*	
MDD diagnosis (<i>n</i>)	28	0		<0.001*

Abbreviations: TNF α = tumor necrosis factor, IL1 β = interleukin-1 β , IL6 = Interleukin-6, IFN γ = interferon γ , CRP = C-reactive protein, GABR = global arginine bioavailability ratio, NSAIDs = non-steroidal anti-inflammatory drugs, CAPS = Clinician Administered PTSD Scale, ETI = Early Trauma Inventory, BDI-II = Beck Depression Inventory-II, PANAS = Positive and Negative Affect Schedule, MDD = Major Depressive Disorder.

* $p < 0.05$.

^a Biochemicals raw data are re-scaled to have a median equal to 1.

^b This value refers to ANOVA analysis using Blom-transformed values.

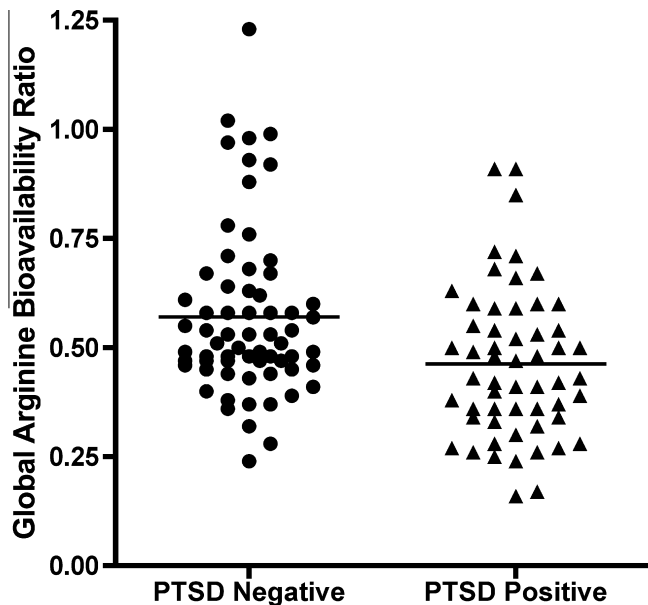


Fig. 2. Unadjusted raw (i.e. not Blom-transformed) GABR values in PTSD subjects and in controls.

PTSD had a significantly higher total pro-inflammatory score ($p = 0.007$) than controls, as well as significantly higher concentrations of certain cytokines (i.e. $\text{TNF}\alpha$, IL6 and CRP), as previously reported in a study using a partially overlapping sample (Lindqvist et al., 2014). Considering the individual components of the GABR separately, PTSD subjects in comparison to controls had significantly higher ornithine ($p = 0.029$), significantly lower arginine ($p = 0.009$) and non-significantly different citrulline ($p = 0.529$) (as reported in Mellon et al. (2015), a study using a partially overlapping sample). As expected, subjects with PTSD compared to controls had significantly higher scores on BDI-II, CAPS current, CAPS lifetime and PANAS negative clinical scales ($p < 0.001$), as well as nearly significant more early traumatic experiences ($p = 0.054$) and significantly lower scores on PANAS positive subscale ($p < 0.001$).

3.2. Comparison of GABR scores between groups (PTSD subjects vs controls)

The GABR value was 0.46 ± 0.17 in PTSD subjects and 0.57 ± 0.19 in combat controls. One-way ANOVA determined highly significant group differences between PTSD subjects and controls ($F[1, 120] = 11.92, p = 0.001$) (Fig. 2). An extended model using age and BMI as covariates did not alter the significance of this result ($F[1, 114] = 7.324, p = 0.008$); in this analysis, BMI accounted for more variance ($F = 4.825; p = 0.030$) than age ($F = 1.478; p = 0.227$). The scatterplot showed in Fig. 2 suggests that there is one PTSD-negative individual with a GABR value substantially higher than average, potentially driving the inter-group difference. Therefore, we re-ran the ANOVA and the ANCOVA analyses excluding that subject, and the inter-group differences were still highly significant ($p \leq 0.01$).

We assessed the possible contribution of MDD diagnosis in three ways. First, we compared three groups by one-way ANOVA: PTSD subjects without comorbid MDD (GABR value: 0.45 ± 0.20), PTSD subjects with comorbid MDD (GABR value: 0.47 ± 0.15), and healthy controls (GABR value: 0.57 ± 0.19). The overall group effect was significant ($F[2, 120] = 6.32, p = 0.002$). Post-hoc Bonferroni analysis showed that healthy controls had a GABR value significantly higher than subjects with PTSD only (without

Table 2

Correlations of GABR with inflammatory markers and psychopathological measures in the unadjusted and adjusted (ie. Covarying for age and BMI) models.

	Unadjusted model		Adjusted model	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Total pro inflammatory score	-0.18	0.081	-0.14	0.189
IL6	-0.20	0.044*	-0.16	0.103
$\text{TNF}\alpha$	-0.23	0.021*	-0.20	0.046*
CRP	-0.17	0.061	-0.07	0.437
IL1 β	0.05	0.960	-0.02	0.863
IFN γ	-0.10	0.320	-0.16	0.109
CAPS current	-0.29	0.001*	-0.24	0.008*
CAPS lifetime	-0.32	<0.001*	-0.27	0.003*
ETI	-0.18	0.045*	-0.171	0.067
PANAS negative	-0.25	0.006*	-0.22	0.017*
PANAS positive	0.15	0.092	0.12	0.212
BDI-II	-0.17	0.065	-0.095	0.314

Abbreviations: IL6 = interleukin-6, $\text{TNF}\alpha$ = tumor necrosis factor, CRP = C-reactive protein, IL1 β = interleukin-1 β , IFN γ = interferon γ , CAPS = Clinician Administered PTSD Scale, ETI = Early Trauma Inventory, PANAS = Positive and Negative Affect Schedule, BDI-II = Beck Depression Inventory-II.

* $p < 0.05$.

comorbid MDD) ($p = 0.004$) and near significantly higher than subjects with both PTSD and MDD ($p = 0.075$); the GABR value was not significantly different between PTSD subjects with and without comorbid MDD ($p = 1.000$). Second, we performed an ANCOVA comparing GABR values in PTSD subjects and controls using MDD diagnosis as a covariate, and the significant inter-group differences remained ($F[1-117] = 9.41; p = 0.003$). Third, in an extended model, we used age, BMI and MDD diagnosis as covariates; this led again to significant inter-group (PTSD vs controls) GABR differences ($F[1-113] = 6.78; p = 0.010$).

We assessed the possible contribution of antidepressant use in three ways. First, we compared three groups by one-way ANOVA: PTSD subjects taking antidepressants (GABR value: 0.39 ± 0.11), PTSD subjects not taking antidepressants (GABR value: 0.49 ± 0.18), and healthy controls (with the exclusion of the only two healthy controls taking antidepressants; GABR value: 0.57 ± 0.19). The overall group effect was significant ($F[2, 118] = 7.78, p = 0.001$). Post-hoc Bonferroni analysis showed that healthy controls had a GABR value significantly higher than subjects with PTSD taking antidepressants ($p = 0.001$) and near significantly higher than PTSD subjects not taking antidepressants ($p = 0.087$); the GABR value was not significantly different between PTSD subjects who did or did not use antidepressants ($p = 0.149$). Second, we performed an ANCOVA comparing GABR values in PTSD subjects and controls using antidepressants as a covariate, and the significant inter-group differences remained ($F[1-118] = 6.67; p = 0.011$). Third, in an extended model, we used age, BMI and antidepressant use as covariates; this led to near-significant inter-group (PTSD vs controls) GABR differences ($F[1-113] = 3.89; p = 0.051$).

3.3. Correlations of GABR with inflammatory scores and clinical assessments

GABR score tended to be negatively correlated with total pro inflammatory score ($r = -0.18, p = 0.081$). Given the trend towards significance, we conducted exploratory analyses on individual (Blom-transformed) cytokines, showing that GABR was significantly inversely correlated with IL6 ($r = -0.20, p = 0.044$) and $\text{TNF}\alpha$ ($r = -0.23, p = 0.021$), nearly significantly inversely correlated with CRP ($r = -0.17, p = 0.061$) and not correlated with IL1 β ($r = 0.05, p = 0.960$) and IFN γ ($r = -0.10, p = 0.320$). More details are given in Table 2.

The GABR was significantly negatively correlated with CAPS current ($r = -0.29, p = 0.001$), CAPS lifetime ($r = -0.32, p < 0.001$),

ETI ($r = -0.18$, $p = 0.045$), and PANAS negative ($r = -0.25$, $p = 0.006$), near significantly negatively correlated with BDI-II ($r = -0.17$, $p = 0.065$) and near significantly positively correlated with PANAS positive ($r = 0.15$, $p = 0.092$) (Table 2).

An extended model using age and BMI as covariates also led to similar results, with the exception of IL6 and ETI losing their significant association with GABR (Table 2). Additionally, adding antidepressant use or MDD diagnosis as covariates led to similar results.

4. Discussion

This is the first study assessing the global arginine bioavailability (i.e. the GABR value, a marker of NO synthetic capacity *in vivo*) in individuals with PTSD and its correlation with markers of inflammation and clinical measures.

Our first finding was a significantly lower GABR value in combat-exposed male PTSD subjects compared to combat-exposed male psychiatrically healthy controls (both without and with adjustment for age and BMI) ($p < 0.01$) (Fig. 2).

It was not possible to fully assess the effect of MDD *per se* on GABR, since MDD only occurred in subjects with comorbid PTSD. We attempted to dissect out the contribution of depression in three ways: (i) performing ANOVA analyses with pair-wise post-hoc testing comparing the GABR value in psychiatrically healthy controls, subjects having PTSD alone and subjects having both PTSD and MDD; (ii) adding MDD diagnosis as a covariate; (iii) performing an extended model with age, BMI and MDD diagnosis as covariates. Overall, these results suggested that comorbid MDD does not play a substantial role in explaining the between-group GABR differences, as (i) healthy controls had a GABR value significantly higher than subjects with PTSD only (without comorbid MDD) ($p = 0.004$), (ii) GABR was not significantly different between PTSD subjects with and without comorbid MDD ($p = 1.000$), and (iii) adding MDD as a covariate (alone or in combination with age and BMI) led to significant GABR inter-group (PTSD vs controls) differences $p \leq 0.010$.

Similarly, it was not possible to fully assess the effect of antidepressants *per se* on GABR, since antidepressants were used almost exclusively by subjects who had PTSD (only two of the psychiatrically healthy controls used antidepressants). We attempted to dissect out the contribution of antidepressants in three ways: (i) performing ANOVA analyses with pair-wise post-hoc testing comparing the GABR value in psychiatrically healthy controls, PTSD subjects taking antidepressant and PTSD subjects not taking antidepressants; (ii) adding antidepressants use as a covariate; and (iii) performing an extended model with age, BMI and antidepressants use as covariates. Overall, these results suggested that antidepressants may play some role in explaining the between-group GABR differences, as (i) GABR values were significantly different in controls compared to patients taking antidepressants ($p = 0.001$), but only near-significantly different in controls compared to patients not taking antidepressants ($p = 0.087$), and (ii) when age, BMI and antidepressants use were entered as covariates the significance of GABR inter-group difference (PTSD vs controls) decreased ($p = 0.051$). This is consistent with previous evidence that certain antidepressants may affect NO biosynthesis (Kraus et al., 2011; Khoshnoodi et al., 2015). However, it is unlikely that antidepressants accounted for the full effect because (i) GABR values were not significantly different in patients who did and did not take antidepressants ($p = 0.149$), and (ii) when antidepressants use alone was used as a covariate, the GABR inter-group difference (PTSD vs controls) was still significant ($p = 0.011$).

Several mechanistic hypotheses might help explain the decreased GABR score in PTSD in comparison with controls. From

a molecular point of view, the reduced NO synthetic capacity in PTSD subjects (i.e. decreased GABR value) could be related to an increased activity of arginase, the enzyme that converts arginine to ornithine (Huynh and Chin-Dusting, 2006). This is a more likely explanation of the lower GABR values in PTSD than an explanation involving altered NOS activity (Fig. 1) since we observed significantly increased ornithine but no significant changes in citrulline in PTSD subjects (Table 1). In addition, previous studies demonstrated that Reactive Oxygen Species (ROS), which reportedly can be increased in PTSD (Cepnija et al., 2011; Wilson et al., 2013), may increase arginase activity possibly leading to a limitation of NO formation through increased arginine consumption (Chandra et al., 2012; Morris et al., 2004; Moss et al., 2004; Tang et al., 2009, 2013) and subsequent reduced substrate availability to NOS enzymes (Chandra et al., 2012; Morris et al., 2004; Moss et al., 2004; Tang et al., 2009, 2013).

From a neuroendocrine point of view, preclinical studies suggested that both catecholamines and glucocorticoids are potent inhibitors of NO production (Chang and Liu, 2000; Lopez-Figueroa et al., 1998; Riedel, 2000). Dopamine, norepinephrine and epinephrine, in fact, have been reported to inhibit microglial NO production (Chang and Liu, 2000). Glucocorticoids can affect the transcription and activity of NOS enzymes (Lopez-Figueroa et al., 1998). Therefore, altered neurotransmitter or neuroendocrine patterns often observed in PTSD (Meewisse et al., 2007; Southwick et al., 1999) might contribute to reduced NO synthetic capacity.

Also, several PTSD-associated phenotypes (e.g. metabolic dysregulation, increased BMI or visceral adiposity, diabetes, insulin resistance [Levine et al., 2014]) can themselves be associated with lowered NO production (Huang, 2009; Siervo et al., 2011). This is consistent with the observed association between decreased GABR and PTSD as well as with the significant inverse correlations between BMI and GABR found in our sample. However, the between-groups differences in GABR values remained significant even after covarying for BMI, suggesting that increased BMI did not fully explain such between-group differences.

Our second finding was a significant or near significant inverse correlation between GABR score and several markers of inflammation including total pro inflammatory score, IL6, TNF α and CRP (Table 2). However, after adjusting for age and BMI, only TNF α was still significantly correlated with GABR.

There is a large body of evidence that NO is involved in several inflammatory disorders (Cirino et al., 2006). It has been shown that NO can be pro-inflammatory or anti-inflammatory (Cirino et al., 2006). For these reasons NO has been described as “double edge sword mediator” and this phenomenon is often referred to as the “NO paradox” (Cirino et al., 2006). Our findings of inverse correlations between GABR scores and markers of inflammation support a possible anti-inflammatory action of NO, although in our correlational analyses the direction of causality, if any, is not known. According to preclinical evidence, the anti-inflammatory action of NO might be mediated by the NO-related modulation of nuclear transcription factor-kB (NF-kB) (Colasanti and Persichini, 2000; Laroux et al., 2001) and subsequent inhibition of the expression of genes involved in inflammatory states (e.g. chemokines, adhesion molecules, interleukins, and cyclooxygenase-2) (Colasanti and Persichini, 2000; Laroux et al., 2001). The combination of decreased NO synthetic capacity and increased proinflammatory cytokines observed in our sample might be especially deleterious as a risk factor for cardiovascular disease and other PTSD-associated somatic disturbances (Levine et al., 2014).

Our third finding was a significant inverse correlation of GABR score with scales rating current or lifetime PTSD symptoms severity, childhood traumatic experiences and negative affectivity (Table 2). While the correlation with ETI was partially explained

by age and BMI, correlations with CAPS current or lifetime and PANAS negative were not. Psychopathological symptoms, childhood traumatic experiences and negative affectivity have been shown to negatively affect individuals' health in several different ways ranging from a maladaptive modulation of cellular physiology (Epel et al., 2004; Insel and Quirion, 2005; Shalev et al., 2013) to a negative influence on health behaviors adoption (Scott, 2014; Tindle et al., 2010). Our data preliminarily suggest that the modulation of NO synthetic capacity may be an additional mechanism by which these psychological dimensions can be associated to worse cellular and health outcomes. Overall, the association between reduced GABR and more severe psychopathology might contribute to explain the poor cellular and health outcomes often seen in PTSD subjects (Levine et al., 2014).

Our results replicate those of the only previous study that measured NO concentration *in vivo* in a sample of people with a stress-related disorder (i.e. ASD) (Yeh et al., 2002). As reviewed in the introduction, in this study Yeh et al. found (i) male military personnel affected by ASD had lower plasma NO levels than controls, and (ii) a significant inverse correlation between the severity of stress-related symptoms and the plasma concentration of NO in ASD subjects (Yeh et al., 2002). Our study, however, differs from this previous study as (i) subjects were diagnosed with PTSD rather than ASD; (ii) we explored affectivity and childhood traumatic experiences in addition to PTSD-related symptoms, (iii) we explored inflammatory markers; (iv) we did not directly measure NO but rather we calculated an approximation of NO synthetic capacity; and (v) the control group in that study was not trauma-exposed.

4.1. Limitations and strengths

Limitations of the present study include (i) our use of an all male study sample; future studies, including an ongoing one by our group, will be needed to investigate metabolic parameters in combat exposed females. (ii) Since this was a cross-sectional study based on single time-point blood and behavioral measurement, we cannot assess any causal relationships or variability in the measures over time. In addition, as it was a single time point measure, we were not able to assess moment-to-moment variability in the measures. (iii) The CAPS lifetime measure relies on subjective recall. (iv) We assessed a relatively large number of molecular and behavioral measures, and we did not correct for multiple comparisons; therefore, these results are exploratory/hypothesis-generating. (v) We did not directly measure NO but rather we calculated an approximation of NO synthetic capacity. (vi) Serum/plasma markers of NO may or may not correspond to brain markers which may be more linked to PTSD. (vii) We were not able to unequivocally evaluate the potential contribution of MDD and antidepressant use on GABR values, although our data suggest that PTSD diagnosis had an independent role in explaining GABR differences.

Among the strengths, (i) to our knowledge this is the first study assessing the global arginine bioavailability in individuals with PTSD and its correlation with markers of inflammation and clinical measures, (ii) the sample was clinically well-characterized, relatively large and comprised of relatively young veterans (this is important since age-related illnesses can pose significant confounds in studies of psychiatric disorders in older subjects), (iii) all blood samples were drawn at fasting and at the same time of day, (iv) all study participants (PTSD subjects and controls) had been exposed to combat, allowing us to control for the non-specific effects of serving in the military and experiencing combat. However, use of this sample as a control group may have resulted in selecting a particularly resilient sample. (v) In an extended model we statistically controlled for potential confounds such as age and BMI.

4.2. Conclusion

In conclusion, the present study provides the first evidence that the global arginine bioavailability, a marker of NO synthetic capacity *in vivo*, is decreased in veterans with PTSD and is negatively associated with markers of inflammation as well as with measures of PTSD symptom severity, negative affectivity and childhood adverse experiences. These findings add to the accumulating evidence that specific cellular dysfunction is associated with the comprehensive symptomatology of PTSD. Our findings may also help to explain the higher burden of cardiometabolic disturbances seen in the disorder and contribute to expand our view of PTSD from a purely mental illness to an illness with important somatic manifestations. This could, in turn, lead to novel treatment options for both the psychiatric and somatic aspects of this condition.

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