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Relations of combat stress and posttraumatic stress disorder to 24-h plasma and cerebrospinal fluid interleukin-6 levels and circadian rhythmicity

Agorastos Agorastos[a,b], Richard L. Hauger[a,c,d], Donald A. Barkauskas[f], Imanuel R. Lerman[a,e], Tobias Moeller-Bertram[c,e], Clara Snijders[g], Uzair Haji[c], Piyush M. Patel[c,e], Thomas D. Geracioti[h], George P. Chrousos[i], Dewleen G. Baker[a,c,d,*]

[a] VA Center of Excellence for Stress and Mental Health, San Diego, CA, USA
[b] Department of Psychiatry, Division of Neurosciences, School of Medicine, Faculty of Medical Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece
[c] VA San Diego Healthcare System, San Diego, CA, USA
[d] Department of Psychiatry, University of California, San Diego (UCSD), CA, USA
[e] Department of Anesthesiology, University of California, San Diego, San Diego, CA, USA
[f] Department of Preventive Medicine, Keck School of Medicine, University of Southern California (USC), Los Angeles, CA, USA
[g] School for Mental Health and Neuroscience (MHeNS), Department of Psychiatry and Neuropsychology, Faculty of Health, Medicine and Life Sciences, Maastricht University, Maastricht, the Netherlands
[h] University of Cincinnati Medical Center, Department of Psychiatry and Neurobehavioral Sciences, Cincinnati, OH, USA
[i] First Department of Pediatrics, School of Medicine, National and Kapodistrian University of Athens, "Aghia Sophia" Children's Hospital, Athens, Greece

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ABSTRACT

Background: Acute and chronic stress can lead to a dysregulation of the immune response. Growing evidence suggests peripheral immune dysregulation and low-grade systemic inflammation in posttraumatic stress disorder (PTSD), with numerous reports of elevated plasma interleukin-6 (IL-6) levels. However, only a few studies have assessed IL-6 levels in the cerebrospinal fluid (CSF). Most of those have used single time-point measurements, and thus cannot take circadian level variability and CSF-plasma IL-6 correlations into account.
Methods: This study used time-matched, sequential 24-h plasma and CSF measurements to investigate the effects of combat stress and PTSD on physiologic levels and biorhythmicity of IL-6 in 35 male study volunteers, divided in 3 groups: (PTSD = 12, combat controls, CC = 12, and non-deployed healthy controls, HC = 11).
Results: Our findings show no differences in diurnal mean concentrations of plasma and CSF IL-6 across the three comparison groups. However, a significantly blunted circadian rhythm of plasma IL-6 across 24 h was observed in all combat-zone deployed participants, with or without PTSD, in comparison to HC. CSF IL-6 rhythmicity was unaffected by combat deployment or PTSD.
Conclusions: Although no significant group differences in mean IL-6 concentration in either CSF or plasma over a 24-h timeframe was observed, we provide first evidence for a disrupted peripheral IL-6 circadian rhythm as a sequel of combat deployment, with this disruption occurring in both PTSD and CC groups. The plasma IL-6 circadian blunting remains to be replicated and its cause elucidated in future research.

1. Introduction

Psychoneuroimmunological research offers mounting evidence of a bi-directional communication between the human immune and stress systems both centrally and peripherally, i.e., through the hypothalamic-pituitary-adrenal axis (HPA axis) and the autonomic nervous system (ANS) (Webster Marketon and Glaser, 2008). Acute and chronic stress can lead to a disrupted interplay between these two systems with consequent over-activation and/or dysfunctional regulation of the systemic and local immune response (Dhabhar, 2014; Glaser and Kiecolt-Glaser, 2005; Menard et al., 2017).

Accordingly, accumulating evidence suggests peripheral immune dysregulation, low-grade inflammation, increased neuroinflammatory responses and altered immune-related gene expression to be associated with posttraumatic stress disorder (PTSD) (Breen et al., 2018; Deslauriers et al., 2017; Gill et al., 2009; Hoge et al., 2009; Lerman et al., 2016; Miller et al., 2018;O'Toole and Catts, 2008; Passos et al., 2015; Smith et al., 2011), while inflammation-related biomarkers have

* Corresponding author at: Department of Psychiatry, University of California San Diego, 9500 Gilman Drive (0603V), La Jolla, CA, 92093-0603V, USA.
E-mail address: dgbaker@ucsd.edu (D.G. Baker).

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Table 1
Demographic and psychometric measures and their group differences in PTSD patients, combat and healthy controls.

Measures	PTSD (n = 12)	CC (n = 12)	HC (n = 11)	F	p		Post-hoc
Age	27.3 (4.6)	31.7 (8.9)	30.9 (6.1)	1.38	.267		n.s.
BMI	25.6 (2.8)	26.4 (2.1)	23.0 (3.3)	4.80	**.015**	*	PTSD = CC > HC
CES	23.3 (7.6)	19.4 (5.4)	N/A	2.10	.161		n.s.
GAF	65.9 (6.0)	77.1 (8.6)	84.5 (9.4)	15.22	< **.001**	***	PTSD < CC < HC
BDI	15.5 (7.9)	3.7 (4.2)	.7 (1.6)	25.12	< **.001**	***	PTSD > CC = HC
HDRS	6.6 (3.5)	1.4 (2.9)	N/A	14.63	**.001**	**	PTSD > CC
CAPS	62.1 (8.6)	14.1 (14.3)	N/A	95.00	< **.001**	***	PTSD > CC
CTQ	40.5 (10.4)	39.1 (14.6)	34.7 (9.7)	.75	.480		n.s.
DES	19.6 (16.0)	4.3 (2.6)	2.7 (2.0)	11.26	< **.001**	***	PTSD > CC = HC

Values are total score means (*SD*). Psychometric scores report total scores. Age is reported in years; BMI: Body Mass Index (kg/m^2); CES: Combat Exposure Scale; GAF: Global Assessment of Functioning; BDI: Beck Depression Inventory II; HDRS: Hamilton Depression Rating Scale; CAPS: Clinician Administered PTSD Scale; CTQ: Childhood Trauma Questionnaire; DES: Dissociation Experience Scale. *p < 0.05; **p < 0.01; ***p < 0.001.

been recently shown to be putative risk factors for PTSD development (Breen et al., 2015; Daskalakis et al., 2016; Eraly et al., 2014; van Zuiden et al., 2011). In particular, recent gene expression studies provide growing evidence for innate immune dysregulation in PTSD and a specific (i.e., comorbidity-independent) role of cytokines in the pathophysiology of the disorder (Glatt et al., 2013; Guardado et al., 2016; Tursich et al., 2014; Tylee et al., 2015). A recently reported positive association between both mitogen-stimulated T-cell cytokine and innate cytokine production and PTSD symptoms (Smid et al., 2015) suggests a direct effect of cytokine production in stress sensitization, while post-trauma expression of pro-inflammatory cytokines in PTSD patients is likely to be regulated by genotype and environmentally-driven epigenetic effects (Bam et al., 2016; Zhou et al., 2014).

Interleukin-6 (IL-6) is a pleiotropic, pro-inflammatory cytokine with a known circadian rhythm (Agorastos et al., 2014a; Papanicolaou and Vgontzas, 2000) commonly measured in stress studies, as peripheral (blood, adipocyte and fibroblast-derived) IL-6 has been shown to be elevated under physical and psychological stress (Breuninger et al., 1993; Dowlati et al., 2010; Mohamed-Ali et al., 2001; Pace et al., 2006; Papanicolaou et al., 1996; van Gool et al., 1990; Zhou et al., 1993). Many research papers, systematic reviews and meta-analyses have found IL-6 plasma/serum levels and peripheral blood mononuclear cell (PBMC) IL-6 production to be higher in PTSD (Gill et al., 2008; Gill et al., 2009; Gola et al., 2013; Hammad et al., 2012; Hoge et al., 2009; Maes et al., 1999; Passos et al., 2015; Sutherland et al., 2003; Tucker et al., 2010; Tursich et al., 2014), whereas some find no such association (Baker et al., 2001; Jergovic et al., 2015; McCanlies et al., 2011; Plantinga et al., 2013). Of the two studies that investigated basal central IL-6 levels in the cerebrospinal fluid (CSF) of PTSD patients, Baker et al. (Baker et al., 2001) reported higher mean and median CSF IL-6 levels in Vietnam combat veterans with longstanding PTSD, while Bonne et al. (Bonne et al., 2011) failed to replicate this finding in non-combat trauma exposed civilians with PTSD. Both were single time point studies.

However, to date, most PTSD research has focused on IL-6 measurement in one compartment (i.e., either CSF or peripheral blood), and used single time-point collections, thus precluding the possibility of investigating circadian/ultradian variability and differential IL-6 levels across the blood brain barrier (BBB) (Agorastos et al., 2014a; Izawa et al., 2013; Lissoni et al., 1998; Nilsonne et al., 2016; Vgontzas et al., 2005). Given that immune system reactivity follows circadian rhythms imposed by the interplay of central (suprachiasmatic nucleus, SCN) and peripheral clocks (e.g., intrinsic molecular clocks of immune cells) (Keller et al., 2009; Scheiermann et al., 2013), diurnal rhythmicity of IL-6 secretion across the BBB, as shown in previous studies (Agorastos et al., 2014a; Vgontzas et al., 2005), is of particular interest.

The main objective of this study was to investigate and further characterize peripheral and central IL-6 concentrations and biorhythmicity by assessing time-matched, sequential plasma and CSF IL-6 measurements over a 24 h timeframe in deployed, combat-exposed participants with and without PTSD, and civilian non-traumatized controls. Given prior study results, we hypothesized that PTSD participants would show higher CSF and plasma IL-6 levels across 24 h compared to the combat and civilian controls, as well as a potentially affected circadian IL-6 rhythmicity.

2. Materials and methods

2.1. Participants

35 male study volunteers participated in this serial CSF and plasma sampling study. The participants were divided into 3 groups: combat veterans with PTSD (n = 12), without PTSD (Combat Controls, CC, n = 12), and healthy male U.S. civilians (Healthy Controls, HC, n = 11). Combat veterans, all of whom were deployed to Iraq or Afghanistan, endorsed similar moderate to high levels of combat exposure (Table 1). For HC group, we used the same healthy civilian study population analyzed and reported in our previous paper (Agorastos et al., 2014a). This study was approved by the UCSD Medical Center Institutional Review Board and the San Diego VA Medical Center Research Committee

Exclusion criteria for all three groups included presence or history of physical co-morbidities, abuse/dependency of alcohol, use of illicit substances, tobacco use, or current use of prescribed or over the counter medications (e.g., antidepressants, glucocorticoid or non-steroid anti-inflammatory agents) for less than five half-lives prior to admission to the clinical research center (CRC), as well as body mass index (BMI) diverging from the norm (18 < BMI < 30). Furthermore, HC and CC were excluded if (previously) diagnosed with a DSM-IV Axis I mental health disorder. All study volunteers were physically healthy, which was confirmed through a thorough physical and neurological examination, blood laboratory tests, urine toxicology screen, chest X-ray and electrocardiogram. Additional physical examinations performed on the afternoon of admission to the CRC ensured absence of any acute clinical manifestations or febrile body temperature.

2.2. Procedures

Study volunteers recruited by verbal or printed advertisement who met phone-screening enrollment criteria, were invited to the laboratory for a study introduction. Following signing of the consent form, the screening assessment for exclusionary criteria, described above, and diagnostic assessments were completed in an office setting. Eligible participants were scheduled for admission to the CRC at 5 p.m. the day prior to study initiation. From this time point on, study participants remained on a standard-meal low monoamine diet (666 calories: 20% protein, 24% fat, and 56% carbohydrates), and received an evening snack of 300 calories. At 8 p.m. on the day of admission to the CRC, an

indwelling venous catheter was placed for blood draws and a standard meal was provided. Participants fasted until 8 a.m. the following day, when a second IV line was placed in the antecubital vein of the non-dominant arm for delivery of a normal saline solution, infused (100 mL/h) throughout the experiment. Immediately afterwards, a 20-gauge catheter was placed in the lumbar subarachnoid space at the L3/4 or L4/5 level by a licensed anesthesiologist and the participants rested until 11 a.m. 24-h fluid collection began with continuous CSF withdrawal into iced test tubes. All fluids were harvested, processed and directly placed on dry ice near the bedside. CSF was withdrawn at a rate of 0.03 ml/minute, and every 30 minutes the 0.9 ml of collected CSF was separated into four 0.225 ml aliquots for permanent storage. Concurrently, every 30 min, blood was withdrawn into 7.5 ml EDTA-coated tubes and immediately processed in a refrigerated centrifuge. The resulting plasma was aliquoted in the same manner. All aliquoted fluids were subsequently stored at −80 °C, until immediately before assay. Sampling lasted for 24 h, at which time the subarachnoid catheter was removed. Vital signs were monitored hourly. Subjects maintained a regular sleep time at around 10 p.m. (lights off) during each study night. Relative silence was preserved in the study room by turning off electronic media and avoiding loud conversations. A 24-h time period starting at 11:30 a.m. was used for this study.

2.3. Measures

Demographic information and (family) history of psychiatric and physical illness were assessed by standardized exploratory clinical interviews. Psychometric assessment was completed by a trained clinician and included the following: Structured Clinical Interview for the DSM-IV-TR Axis I Disorders (SCID-I), Hamilton-Depression Scale (HDRS), Clinician Administered PTSD Scale (CAPS), and Global Assessment of Functioning (GAF). The CAPS was used for PTSD diagnosis using the well-established F1/I2 scoring rule (Weathers et al., 1999), and was validated by SCID-I PTSD module. Self-rate questionnaires were used to assess combat severity (Combat Exposure Scale, CES), history and severity of childhood trauma (Childhood Trauma Questionnaire, CTQ), dissociative symptomatology during the past month (Dissociative Experiences Scale-II (DES) and depression symptoms (Beck Depression Inventory-II, BDI-II).

2.4. Assays

2.4.1. IL-6 measurements in CSF samples

IL-6 concentrations were measured in CSF samples using the Quantikine ELISA kit (regular sensitivity; R&D Systems, Minneapolis, MN). Lower detection limit and linear range were 0.7 pg/ml and 3.1–300 pg/ml, respectively (intra-assay coefficient of variation: 2%; inter-assay coefficient of variation: 4%).

2.4.2. IL-6 measurements in plasma samples

IL-6 concentrations were measured in plasma samples using the Quantikine HS ELISA kit (high sensitivity; R&D Systems, Minneapolis, MN). Lower detection limit and linear range were 0.04 pg/ml and 0.16-10 pg/ml, respectively (intra-assay coefficient of variation: 7%; inter-assay coefficient of variation: 9%).

IL-6 levels greater than the highest standard were diluted with calibrator media to be within the linear working range and re-assayed.

2.5. Statistical analyses

Statistical analyses were carried out for all subjects who had at least one valid observation for CSF and plasma per time point. The analyses were conducted using R, version 2.14.2. An error probability of $p < .05$ was accepted as statistically significant. Values stemming from a normal distribution are reported as mean (standard deviation, SD) and skewed values as median values (min-max). Plasma and CSF IL-6 levels showed a skewed distribution and were therefore $\log_2$ transformed for further analyses. Relations were investigated using the Pearson product-moment correlation coefficient r. Further statistical analysis was done using linear mixed-effects models with response variables ($\log_2$ of the peptide) for individuals with more than 50% of valid observations (PTSD: $n = 12$; CC: $n = 11$; HC: $n = 8$) (Fitzmaurice et al., 2011). The coefficient of variation (CV) was calculated for individuals with more than 50% of valid observations using the form: $CV_{(data)} = SD[\log_{(data)}] \times 100$. Time-ordered relations between plasma and CSF IL-6 were investigated by time-cross-correlation analyses (Fig. 2) similar to prior studies (Agorastos et al., 2014a; Alesci et al., 2005; Crofford et al., 1997). Cross-correlation was computed at various time lags covering the 24-h period, by leading or lagging the concentration-time series of plasma IL-6 relative to the concentration-time series of CSF IL-6 (Venables and Ripley, 2002). The circadian rhythmicity of IL-6 was assessed by utilizing linear mixed-effects models (Fitzmaurice et al., 2011). The fixed effects were age, BMI, patient group, linear trend in time, and 24-, 12-, and 8-h circadian rhythms, plus interaction terms for patient group and each of the time measures. Age was centered by subtracting 30, BMI was centered by subtracting 26, and time 0 represented 11:30 a.m. on the first day. The random effects considered were random intercepts and slopes for each subject, and the residuals in the model were tested for the presence of an autoregressive correlation structure of order 1 (AR(1) correlation) (Fitzmaurice et al., 2011). The necessity of the AR(1) correlation structure plus random intercepts and/or slopes were tested on a model with all potential fixed effects present. Once the correct random effects were determined, the best model for the fixed effects was determined by a stepwise model selection procedure using the Akaike Information Criterion (AIC) to define the best model (Sakamoto et al., 1986). The 24-h circadian component was handled by either entering in or deleting from the model both $\cos(2\pi t/24)$ and $\sin(2\pi t/24)$ simultaneously, and similarly for the 12- and 8-h circadian components. In order to exclude the possibility of the model being skewed by patients with only a few observations, the final model was run with all patients who had at least 20 observations, and the models were compared to assure the coefficients and p-values were qualitatively and quantitatively similar. The circadian rhythms in the final model were calculated as coefficients of the cosine and sine components and translated into amplitude and peak location. The standard errors of the amplitude and peak location were calculated from those of the sine and cosine terms in the final model by the multivariate delta-method.

3. Results

3.1. Participant characteristics

The mean age of all study participants ($n = 35$) was 29.9 ± 6.9 years; mean BMI was 25.1 ± 3.0 kg/m^2. There were no significant group differences in age, combat exposure, CTQ total score (Table 1), or ethnicity, basic laboratory test results, mean pulse, mean systolic and diastolic blood pressure and temperature between the three groups (*data not shown*). Groups did differ significantly with respect to psychometric data, with the PTSD group having significantly higher CAPS, BDI, DES and HDRS scores and significantly lower GAF than the CC and HC group. In addition, both PTSD and CC groups had significantly higher BMI scores than the HC group.

3.2. IL-6 levels in plasma and CSF

A total of 701 plasma (HC: $n = 8$, observations = 163; CC: $n = 11$, observations = 260; PTSD: $n = 12$, observations = 278) and 664 CSF (HC: $n = 10$, observations = 187; CC: $n = 12$, observations = 255; PTSD: $n = 11$, observations = 222) observations over the 24 h timeframe were included in our analyses. Within-subject IL-6 CV ranged from 18.9 to 204.2% in plasma (mean: 75.3% ± 44.5%) and 22.5 to

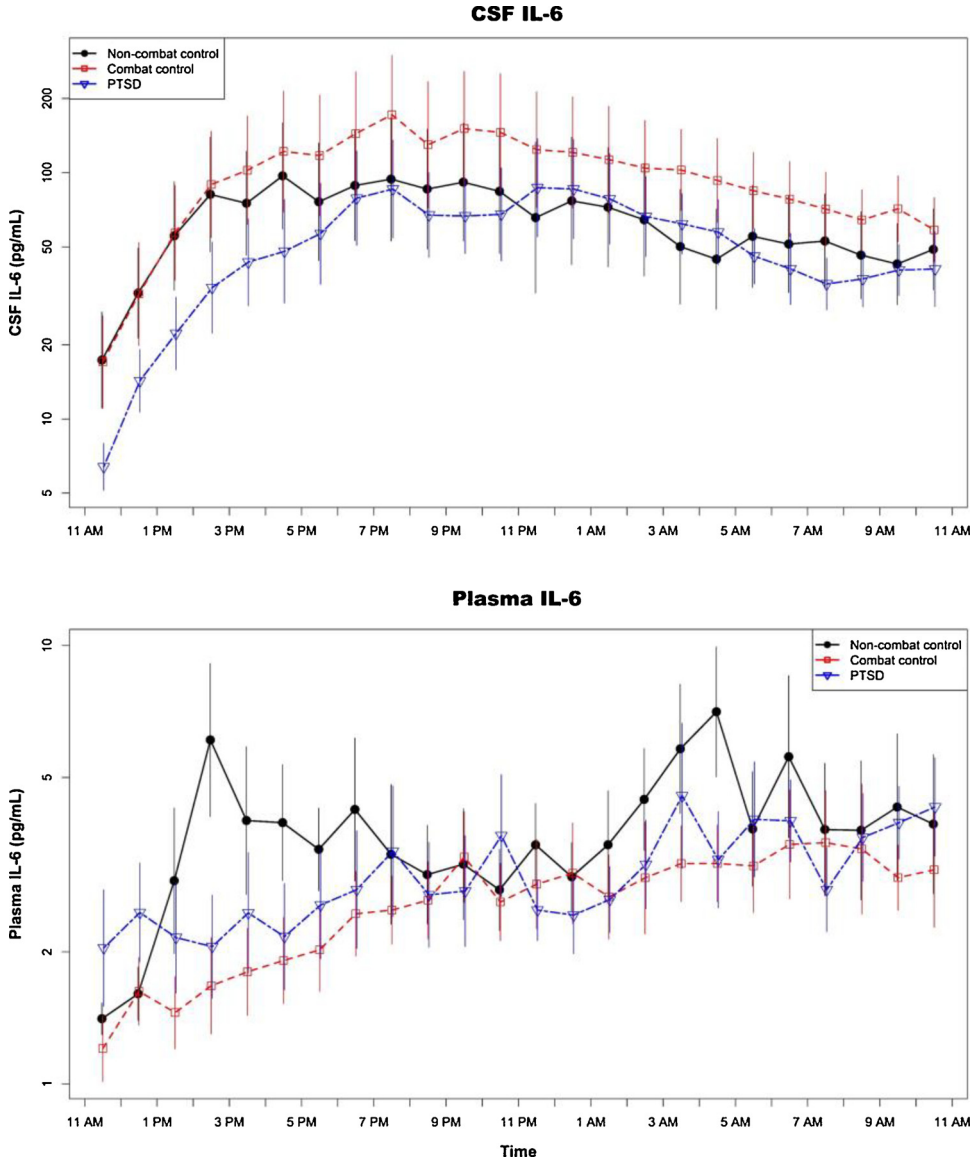

Fig. 1. Geometric means of diurnal cerebrospinal fluid and plasma IL-6 concentrations in the three groups across 24 h.
This figure presents the CSF and plasma IL-6 concentrations across the investigated 24-hour timeframe for the three groups. IL-6 concentrations showed a skewed distribution and were therefore $\log_2$-transformed. The arithmetic means and SEs calculated from log-transformed data were back-transformed to their original scale through exponentiation, so the sample (arithmetic) means on the log scale represent geometric means on the raw scale. Geometric means and SEs were plotted over time with log scaling on the vertical axis.

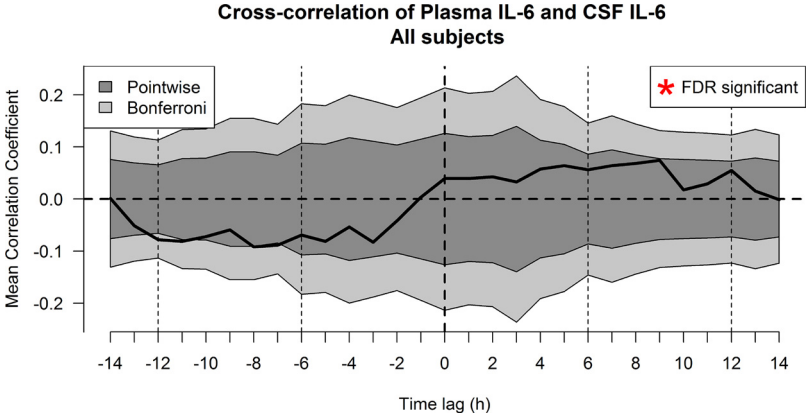

Fig. 2. Cross-correlation analysis between plasma and CSF IL-6 levels over the 24-h study period in all study participants.
Cross-correlation analysis presenting the mean of the individual values of the correlation coefficient R_x for each subject at lag time x (solid line). The dark gray area represents the standard error of the mean (SEM) and indicates the limits of statistical significance for cross-correlation at $p = .05$ level. Significant correlation at any lag time is achieved when the solid line falls outside the dark gray area. The light gray area indicates the limits of statistical significance for cross-correlations after Bonferroni correction. Positive lags mean plasma IL-6 is at later point then CSF IL-6. FDR analysis revealed no potential Type I errors.

89.3% in CSF (mean: 56.1% ± 16.2%). The IL-6 geometric means for plasma and CSF IL-6 for across 24 hours for the three groups are illustrated in Fig. 1. The linear fixed model analysis showed no significant effect of age ($p = .959$), BMI ($p = .284$) or study group (PTSD, CC, HC; $p = .757$) on 24-h plasma IL-6 concentration. For CSF IL-6, the linear fixed model analysis showed no significant effect of age ($p = .403$) or study group (PTSD, CC, HC; $p = .172$) on 24-h CSF IL-6 concentration, but did show a significant effect of BMI ($p = .011$) on CSF IL-6. Further investigation of this finding showed that the effect was mainly driven by the CC group, and that when investigating only those participants with more than 20 observations, the effect disappeared. Additional analyses of the area under the curve (AUC) confirmed that plasma and CSF IL-6 did not differ between groups across the 24 hours after testing for multiple comparisons (*data not shown*). Supplementary correlation analyses revealed no significant correlation of plasma or CSF IL-6 with hemodynamic measures (resting heart rate, systolic and diastolic blood pressure), with any psychometric or combat exposure score in the cohort as a whole, or in any of the three study groups separately (*data not shown*). No correlation pattern between time-matched mean CSF and plasma IL-6 values were observed within subjects (*data not shown*). Cross-lagged correlation analyses between plasma and CSF IL-6 concentrations in the whole sample revealed no correlations within the time frame investigated (positive/negative lag time analysis). The mean of the coefficients of correlation between plasma and CSF IL-6 level-time series at any lag time is presented in Fig. 2.

3.3. Circadian rhythmicity

3.3.1. Plasma

Our results indicated a significant linear trend for all three groups ($p < .001$, respectively) over the 24-h timeframe. The results of the harmonic analysis indicated that plasma IL-6 concentrations showed a significant 12h component ($p = .048$) with distinctive group differences ($p = .010$, Fig. 3). The HC group showed an amplitude of .525 ($SE = .12$) with biphasic peaks at roughly 4:03 p.m. and 4:03 a.m. ($SE = 28$ min) as reported previously (Agorastos et al., 2014a), while both PTSD and CC showed significantly smaller amplitude with blunted peaks [.043 ($SE = .09$) and .048 ($SE = .1$) respectively, n.s. different from zero]. Both the plasma IL-6 24 h component [$p = .049$; amplitude: .19 ($SE = .09$), Fig. 4] and 8 h component [$p = .101$; amplitude: .118 ($SE = .056$)] were without significant group differences ($p = .553$ and $p = .613$, respectively).

3.3.2. CSF

Our results indicated a significant linear trend for all three groups ($p < .001$, respectively) over the 24-h timeframe. The results of the harmonic analysis indicated that CSF IL-6 concentrations showed a significant 24h component ($p < .001$) with no group differences ($p = .153$, Fig. 4). All three groups showed an amplitude of 1.27 ($SE = 0.124$) with peaks at 7:58 p.m. ($SE = 23$ min) as reported previously (Agorastos et al., 2014a). Both the CSF IL-6 12 h component [$p < .001$; amplitude: 0.34 ($SE = 0.065$)] and 8 h component [$p < .001$; amplitude: 0.18 ($SE = 0.043$)] were without significant group differences ($p = .914$ and $p = .975$, respectively).

4. Discussion

In this study, cross-group, time-matched, repeated 24-h plasma and CSF IL-6 measurements were performed to investigate the effects of combat stress exposure and PTSD on overall concentrations and circadian secretion patterns of IL-6 in the peripheral and central compartments in males. The main findings of this study are: i) replication of our prior finding of a physiologic 24-h CSF IL-6 circadian rhythm (Agorastos et al., 2014a) in both CC and PTSD groups, ii) no alteration of CSF 24-h circadian rhythm in the combat-zone deployed (PTSD, CC) participants, as compared to the healthy civilians (HC), iii) blunted peripheral IL-6 circadian rhythm with loss of the biphasic IL-6 pattern in plasma in both the PTSD and CC as compared to the HC group, iv) no significant group differences in IL-6 24-h geometrical mean levels in either CSF or plasma across the 24-h timeframe, and v) no differences in cross-lagged CSF-plasma IL-6 correlations across study groups. To the best of our knowledge, there are no comparable prior research findings in humans to date.

The overall higher IL-6 levels in the CSF as compared to plasma, as well as the diverging circadian IL-6 patterns between both compartments, are in accordance with prior research suggesting local peripheral and central IL-6 production and differential regulation across the blood brain barrier (BBB) and, thus, indicating an independent physiologic role for IL-6 in the two compartments (Agorastos et al., 2014a; De Simoni et al., 1995; Gruol and Nelson, 1997; Juttler et al., 2002; Otten et al., 1994; Reyes and Coe, 1996, 1998). However, the most notable finding of this study is the isolated loss of the biphasic plasma peripheral IL-6 circadian pattern with blunted plasma circadian variability in the combat-zone deployed individuals (CC, PTSD) compared to the non-deployed civilian controls (HC).

Prior lower-animal and human research provides evidence for a particular physiologic link between the circadian system and IL-6 (Agorastos et al., 2014a; Monje et al., 2011; Vgontzas et al., 2005; Vgontzas and Chrousos, 2002; Vgontzas et al., 2002). The production of peripheral IL-6 by immune cells falls under central clock control as a result of nuclear receptor-mediated mechanisms of circulating mediators (i.e. melatonin, glucocorticoids) (Garcia-Maurino et al., 1997; Garcia-Maurino et al., 2000; Giannoulia-Karantana et al., 2006; Guerrero et al., 2000; Srinivasan et al., 2008), while local clocks may also directly drive peripheral IL-6 oscillations (Journiac et al., 2009). The basal rhythmic peripheral secretion of IL-6 is mainly influenced by the intrinsic clock of T-Lymphocytes (i.e. clock and nuclear receptor gene transcription activity such as retinoid-related orphan receptor-alpha, RORα). RORα up-regulates the inflammatory response and enhances IL-6 production through binding to a response element on the promoter of the IL-6 gene (Journiac et al., 2009). In the central nervous system (CNS), circadian pattern of IL-6 could directly modulate neuronal activity and other brain functions (i.e. sleep regulation, thermoregulation, appetite, fatigue) (Bethin et al., 2000; Bluthe et al., 2000; Juttler et al., 2002; Motzkus et al., 2002; Papanicolaou and Vgontzas, 2000; Rohleder et al., 2012; Vgontzas et al., 1999). CNS RORα expression in astrocytes is regulated by IL-6, while RORα and clock gene *Per1* directly activates the IL-6 gene, leading to a bi-directional regulation necessary to maintain central IL-6 basal diurnal patterns and responsivity in inflammatory stress (Journiac et al., 2009; Sugimoto et al., 2014). Interestingly, a genome-wide association study in PTSD identified the RORα gene as a significant risk focus (Logue et al., 2013). Furthermore, the BBB may additionally affect the circadian properties of IL-6 in the CNS, as BBB also displays a circadian rhythm of function and permeability (Nakazato et al., 2017; Tumani et al., 2017; Zhang et al., 2018), that can be altered by chronodisruption (Pan and Kastin, 2016).

In contrast to the blunted IL-6 peripheral circadian rhythm in the previously combat-zone deployed groups, we find no other significant differences in either 24-h CSF or plasma concentrations, or in IL-6 cross-lagged correlations across our study groups (PTSD, CC and HC). Thus, we fail to replicate findings of increased plasma IL-6 levels in PTSD or after trauma exposure (Gill et al., 2008; Gill et al., 2009; Gola et al., 2013; Hammad et al., 2012; Hoge et al., 2009; Maes et al., 1999; Passos et al., 2015; Sutherland et al., 2003; Tucker et al., 2010; Tursich et al., 2014), or our earlier single time point CSF IL-6 finding in Vietnam veterans (Baker et al., 2001). These disparate results may be due to a number of factors, such as differences in study cohorts which may affect immune properties (e.g., chronic PTSD in Vietnam veteran group, differences in smoking status and/or physical or psychiatric comorbidity) or, most likely, in study design (single time point versus 24-h sequential sampling). The current study enrolled participants with relatively

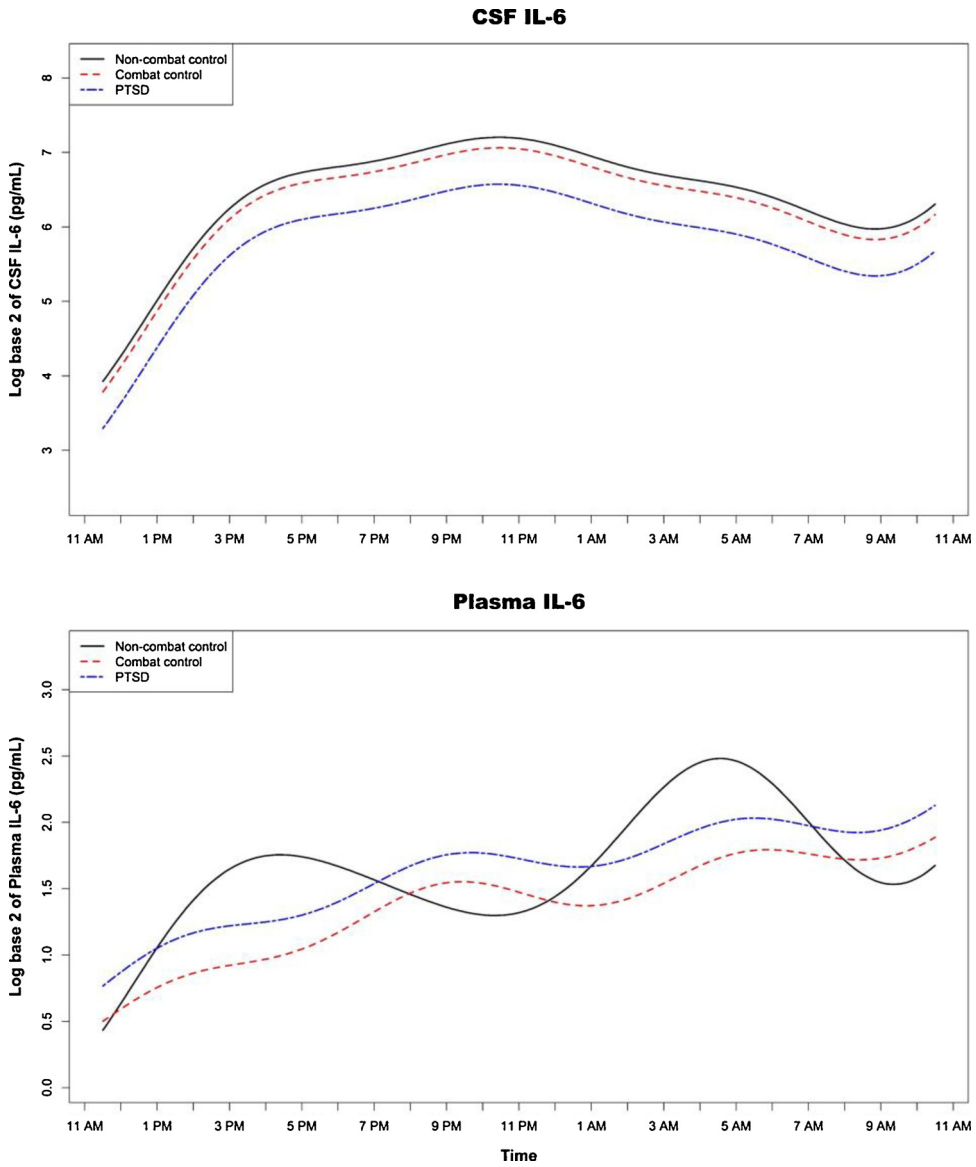

Fig. 3. Linear/circadian trend for Log_2 of cerebrospinal fluid and plasma IL-6 concentrations in the three groups across 24 hours.
This figure presents the time-dependent portion of the final models illustrating the linear trend and circadian rhythmicity of plasma and CSF IL-6 concentrations across the investigated 24-hour timeframe in the three groups separately plotted over time with log scaling on the vertical axis.

recent trauma exposure and less PTSD symptom chronicity, who were non-smokers with no history of prior substance use or alcohol dependence and were not clinically depressed despite the higher depression scores reported.

Taken together, our results suggest circadian IL-6 patterns across the BBB (in the CNS and periphery) that not only differ in periodicity, but that are differentially affected by deployment and/or combat stress exposure. Differential regulation of rhythmic immune functions between compartments, coordinated by different oscillating clocks on multiple levels could be a possible explanation of our findings and might contribute to a better understanding of the time-related interplay between immune-modulators and the circadian system and its modulation through stress (Keller et al., 2009). Contrary to our hypothesis, dysregulation of peripheral IL-6 physiologic circadian patterns appears to be not specifically PTSD driven, but is observable in all deployed individuals. One hypothesis may be that the likelihood of developing clinical conditions from systemic asynchrony might be possibly influenced by individual resilience factors and post-translational modifications (Kohyama, 2011). An individual's (epi)genetic and environmental risk may thus be subject to modulation by the combined actions of the circadian and stress response systems (Landgraf et al., 2014). Stress-related loss of cellular and systemic rhythmicity, may, thus, alter IL-6 secretion and potentially represent a link between this cytokine and an increased risk for the development of mental health and physical comorbidities (Burgos et al., 2006; Castanon-Cervantes et al., 2010; Elenkov et al., 2005; Nader et al., 2010). While acute stress-induced increases in IL-6 might be useful in maintaining homeostasis, a chronic long-term IL-6 dysregulation is indicative of chronic stress and unfavourable health outcomes (Hansel et al., 2010). Accordingly, the pathophysiologic relevance of the central and peripheral IL-6 circadian secretion patterns in humans is likely to be of major importance in stress-related research on emerging anti-IL-6 treatment options (Nishimoto, 2010).

Some additional limitations of our study merit discussion. Because of its invasive and time intensive nature, our study investigates only a small number of male volunteers. Our findings should thus be replicated in larger study populations and across both genders. Veteran participants (PTSD and CC) were assessed within 5 years of their last deployment. However, while index trauma and deployment history were queried via interview, detailed information on specific time,

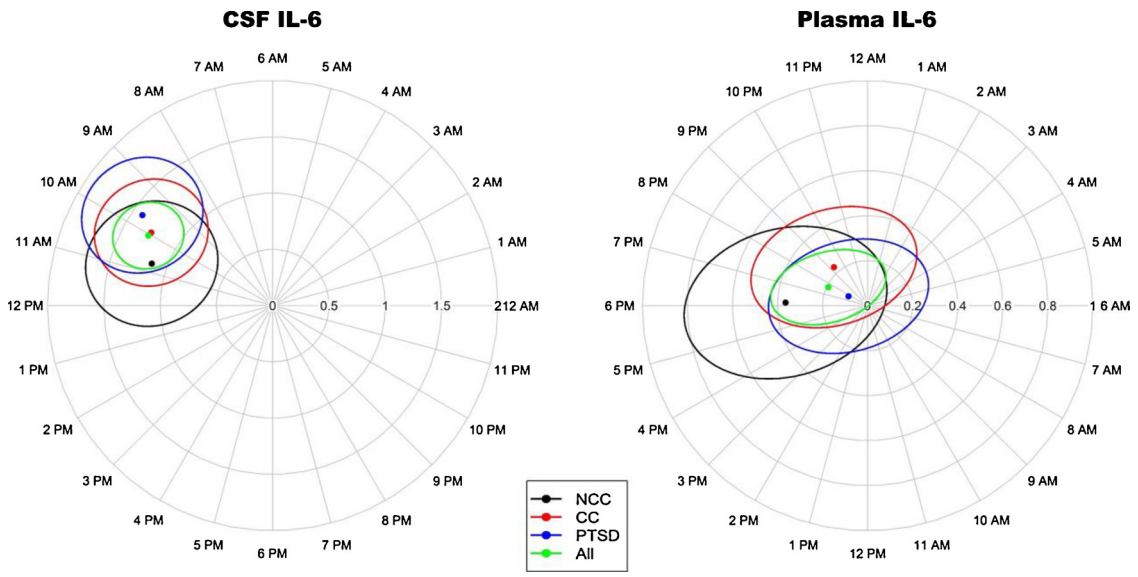

Fig. 4. Circle plots of circadian rhythms for Log_2 IL-6 and 95% confidence intervals in cerebrospinal fluid and plasma across groups.
Circle plots display the location and scale of the peak for the 24-hour circadian rhythm of CSF and plasma IL-6 across groups. The distance from the center represents the amplitude of the peak, and the angle represents the time of day of the peak. The dots represent the point estimates and the ellipses represent 95% joint confidence regions for the time and amplitude combination.

duration and number of previous deployments were not part of the standardized documentation sheet at the time of the study, and is therefore unavailable for a detailed, precise analysis and report. As sleep patterns also affect IL-6 secretion, current unavailability of sleep quality data (e.g., sleep quality questionnaires, sleep-EEG or -activity data) represents another study limitation. Several clinical findings confirm that disturbed day-night cycles and sleep deprivation affect the rhythmic intra-diem oscillations of plasma IL-6 (Cuesta et al., 2016; Monje et al., 2011; Vgontzas and Chrousos, 2002; Vgontzas et al., 1999; Vgontzas et al., 2003; Vgontzas et al., 2002) and generate a functional gap between peripheral circadian IL-6 secretion and immune cell numbers in blood, which results in dysregulated immune cell responsiveness (but not cell-number rhythmicity) and altered IL-6 circadian secretion (Adams et al., 2013; Cuesta et al., 2016). Nevertheless, all subjects were well matched and extremely carefully selected to minimize the probability of medical (medication use, depression) and behavioural (substance, tobacco, alcohol use) confounders, while our 24-h multiple-time point collection allowed us observation across a large number data points, thus increasing statistical power. With respect to some missing data, the harmonic analysis used is functionally equivalent to that employed by Vgontzas et al. (Vgontzas et al., 2005; Vgontzas et al., 1999), except that the linear mixed model can better handle missing data. Furthermore, although the positive correlation of CSF IL6 levels with BMI did not hold up in additional statistical analyses, it may be worth mentioning that latest research has identified shared genetic pathways (e.g., rs10242595 polymorphism) that strongly affect the association between IL-6 and BMI obesity, accounting for the IL-6 responsiveness to diet and life-style factors (Amaral et al., 2015; Andersson et al., 2010). Finally, our study does not provide any information on the concentration of soluble IL-6 receptor or receptor-bound IL-6, both of which may critical variables in understanding IL-6 physiology, in both compartments (Rose-John et al., 2007). Similarly, the absence of environmentally driven epigenetic markers, circadian glucocorticoid levels, T-cell related RORA-α expression and other biological biomarkers could have added to the interpretations of the study findings, however were not part of the initial study protocol. Nevertheless, if replicated in future larger-scale studies, circadian rhythmicity of peripheral IL-6 levels could represent a novel diagnostic biomarker for stress-related research.

4.1. Conclusions

Our study provides first evidence in humans for a significantly blunted circadian rhythm of plasma IL-6 after deployment to a combat zone, while CSF IL-6 circadian patterns remain unaffected. These findings underline the major importance of the circadian system along with the immune and stress systems in the potential development of pathophysiology, and highlight the possible temporal asynchrony in brain and body of stress-exposed individuals (Agorastos et al., 2014b; Dayan et al., 2017). Further understanding of the mechanisms susceptible to circadian dysregulation of the immune system following stress and how it interfaces with stress systems could be valuable in enabling innovative psychochronobiological preventive strategies and treatment possibilities in high-risk trauma-exposed populations (Agorastos and Linthorst, 2016). Detecting and eventually normalizing immune activation may potentially complement strategies to prevent progression of combat stress-related symptoms.

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Contributors

DGB designed the study and wrote the protocol. RLH, IRL, TMB and UH collected the data. AA and CS managed all literature searches. AA, DGB, RLH and DAB had access to the raw data, AA and DAB performed all statistical analyses and data interpretation. AA and DGB wrote the first draft of the paper. RLH, PMP, TDG, CS and GPC revised the draft for important intellectual content. All authors have contributed to, read and approved the final version of the manuscript.

Conflict of interest statement

All authors declare no financial or conflict of interest. The opinions and assertions contained herein are the private views of the authors, and do not necessarily reflect the official positions or policies of the

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