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ORIGINAL INVESTIGATION

Sex differences in neurosteroid and hormonal responses to metyrapone in posttraumatic stress disorder

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Abstract
Rationale Mechanisms contributing to sex differences in the regulation of acute stress responsivity and their effect on the increased incidence of posttraumatic stress disorder (PTSD) in women are poorly understood. The reproductive hormone, progesterone, through conversion to allopregnanolone (ALLO), suppresses the hypothalamic pituitary adrenal (HPA) axis and has potent anxiolytic effects. The potential that progesterone and allopregnanolone reactivity modulate HPA axis responses and account for sex differences in PTSD has not been previously examined.
Objective The present study examined the effects of sex and PTSD on adrenocorticotropic hormone (ACTH), progesterone, and allopregnanolone responses to metyrapone and whether progesterone and allopregnanolone reactivity could affect the ACTH response in PTSD.
Methods Healthy medication-free male and premenopausal follicular phase female participants with chronic PTSD (n=43; 49 % female) and controls (n=42; 50 % female) completed an overnight metyrapone challenge and ACTH, progesterone, and allopregnanolone were obtained by repeated blood sampling.
Results The increase in ACTH response to metyrapone was higher in PTSD subjects compared to controls and in women compared to men. Contrary to our initial prediction of an inverse relationship, progesterone and allopregnanolone were positively associated with ACTH. Progesterone and allopregnanolone partially mediated the relationship between PTSD and ACTH.
Conclusions Our findings of increased ACTH to metyrapone in PTSD and in women may reflect heightened hypothalamic CRF hypersecretion. Progesterone and allopregnanolone partially mediated the ACTH response in PTSD. Further characterizing sex differences in these processes will advance our understanding of the pathophysiology of PTSD, and may ultimately lead to better-targeted, more effective treatment.

Keywords Sex differences · Posttraumatic stress disorder · Progesterone · Allopregnanolone · ACTH · Metyrapone

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Background

Traumatic stress exposure that involves threat to life or physical integrity often results in posttraumatic stress reactions in a

proportion of exposed individuals. Posttraumatic stress disorder (PTSD) is estimated to affect approximately 8–10 % of the US general population and 13–22 % of returning US veterans from Iraq and Afghanistan (Boscarino 2006; Dohrenwend et al. 2006; Friedman 2004; Hoge et al. 2004; Kessler 2000; Kessler et al. 1995). Several epidemiological studies have suggested that women develop PTSD at twice the rate of men, despite greater trauma exposure in men (Adamson et al. 2008; Breslau et al. 1998). Preclinical studies have shown that differences in hypothalamic–pituitary–adrenal (HPA) axis regulation and corticotropin releasing factor (CRF) receptor signaling confers stress hypersensitivity in female compared to male rodents (Bangasser and Valentino 2012); however, the mechanisms that contribute to sex differences in the regulation of acute stress responsivity in humans and their effect on the increased incidence of PTSD in women are poorly understood.

PTSD is associated with increases in both extrahypothalamic and hypothalamic CRF neurotransmission (Baker et al. 1999; Bremner et al. 1997, 2003a; Heim et al. 2001; Sautter et al. 2003; Smith et al. 1989). Hypersecretion of CRF by neurons is believed to be an important component of PTSD pathophysiology based on studies that have detected abnormally high CRF levels in the cerebrospinal fluid of PTSD patients, with the highest CRF concentrations being associated with greatest illness severity, suicide, and psychosis (Baker et al. 1999; Bremner et al. 1997; Hauger et al. 2012; Sautter et al. 2003). Furthermore, chronically elevated hypothalamic CRF activity in PTSD is thought to contribute to the blunted adrenocorticotropic hormone (ACTH) response to CRF in PTSD found in most (Bremner et al. 2003a; Heim et al. 2001; Smith et al. 1989), but not all studies (Kellner et al. 2002; Rasmusson et al. 2001). Also, increased CRF may be involved in the increased ACTH and cortisol response to psychological challenge in PTSD (Bremner et al. 2003b; Elzinga et al. 2003; Heim et al. 2000).

Metyrapone has been used diagnostically as a challenge that allows evaluation of the integrity of the HPA axis. Metyrapone blocks cyp11B1 (11β-hydroxylase) and hence blocks the conversion of 11-deoxycortisol to cortisol resulting in reduced cortisol synthesis and increased levels of 11-deoxycortisol and other adrenocortical steroids prior to the inhibitory step. Reduced cortisol concentrations result in removal of glucocorticoid negative feedback to the hypothalamus and pituitary, which can lead to increased hypothalamic CRF drive and the stimulation of pituitary ACTH release. A study of male combat veterans in which metyrapone was administered in the morning and ACTH was assessed at blood draws obtained at 1, 3, and 5 h later found that those with PTSD had a greater ACTH response compared to non-combat exposed controls (Yehuda et al. 1996). In a subsequent study of male Gulf War Veterans, metyrapone was administered in the morning and blood was drawn at intervals from 8:00 a.m. to 4:00 p.m. Gulf War Veterans with PTSD had a significantly higher ACTH response to metyrapone than veterans without PTSD, although they did not differ from non-exposed control subjects (Golier et al. 2009). In two additional studies, one including male combat veterans and one of female civilian trauma survivors, metyrapone was administered in the evening before sleep and ACTH was measured the following morning, in order to avoid disturbing sleep (Neylan et al. 2003; Otte et al. 2007). In both of these studies, subjects with PTSD had a significantly smaller increase in ACTH response compared to PTSD-free controls. However, it is unclear whether the PTSD subjects and controls had the same immediate peak and recovery of the ACTH response soon after metyrapone administration, since blood was drawn 8–9.5 h after the final metyrapone dose. The discrepancy in findings across studies may be due to differences in metyrapone dosage, the timing of ACTH measurement, time of day, and gender.

Growing literature also suggests that HPA axis activity differs between men and women. Several studies have shown that premenopausal women have lower mean cortisol levels compared to men in the same age range (Van Cauter et al. 1996) and smaller HPA responses to psychological stress (Kirschbaum et al. 1999, 1992). A study in which metyrapone was administered at 4 p.m. and every 4 h through noon the next day found that both depressed and control women had a greater ACTH response compared to depressed and control men. Analyses within each gender revealed that depressed women had an increased initial evening ACTH response compared to control women, and that depressed men had an overall decreased ACTH response over the course of 24 h compared to control men (Young and Ribeiro 2006). No studies to date have examined sex differences in ACTH responses to metyrapone in men and women with PTSD.

Studies have also shown that sex differences in HPA axis responses are influenced by aging. Whereas age-related elevations of cortisol during the nocturnal nadir occurs in both men and women, older postmenopausal women also showed increased plasma cortisol levels in the morning acrophase (Van Cauter et al. 1996). Studies have also shown that older women had higher cortisol responses to CRF administration (Greenspan et al. 1993; Luisi et al. 1998), decreased HPA feedback sensitivity to cortisol (Wilkinson et al. 1997), and an increased ACTH and cortisol response to psychological challenge (Seeman et al. 1995, 2001). However, there are also a few studies showing that older postmenopausal women have lower urinary free cortisol in response to mild laboratory stress (Prinz et al. 2000) in addition to lower ACTH and free salivary cortisol responses to acute stress than men (Kudielka et al. 1998). Despite some inconsistencies, the finding of HPA axis differences associated with gender, age, and menopausal status suggests that reproductive hormones may be involved in modulation of the HPA axis response.

The reproductive hormone progesterone is released by the gonads, brain, and adrenals and has strong sedative and anxiolytic effects, provides neuroprotection, and modulates the HPA axis (Deutsch et al. 2013; Melcangi et al. 2014; Patchev et al. 1996; Roy et al. 1999; Soderpalm et al. 2004; Viau and Meaney 1991). Allopregnanolone (ALLO), the primary metabolite of progesterone, is a neurosteroid that has potent anxiolytic effects in the brain via interactions with GABA-A receptors, which may serve to terminate the acute stress response (Bitran et al. 1995; Paul and Purdy 1992; Reddy et al. 2005). Allopregnanolone potentiates inhibitory activity by GABA (Majewska et al. 1986), and intrahypothalamic GABA inhibits CRF secretion (Tsagarakis et al. 1990). Animal studies have shown that allopregnanolone increases after acute stress (Purdy et al. 1991), suppresses CRF-induced anxiety, and inhibits CRF gene transcription and release (Patchev et al. 1994). Progesterone and allopregnanolone administration have also been shown to inhibit ACTH and corticosterone release after stress (Patchev et al. 1996). Progesterone attenuated CRF-enhanced startle and this effect was shown to be mediated by allopregnanolone (Toufexis et al. 2004). The administration of progesterone was associated with an increase in allopregnanolone and with a reduction of anxious behavior in rodents (Bitran et al. 1993).

In humans, alterations in endogenous progesterone and allopregnanolone levels have been implicated in anxiety and mood disorders (Brambilla et al. 2005). Human studies have demonstrated a relationship between progesterone and cortisol levels during stress (Wirth et al. 2007). Administration of CRF and ACTH resulted in increases in allopregnanolone and progesterone in women (Genazzani et al. 1998). Taken together, the animal and human findings support the possibility that progesterone and allopregnanolone are involved both in the initiation as well as in the termination of the acute stress response and in the attenuation of HPA axis activity. In a study of premenopausal women with PTSD, Rasmusson et al. found that cerebrospinal fluid (CSF) concentrations of allopregnanolone were 39 % lower in women with PTSD compared to healthy controls, consistent with a possible protective effect by allopregnanolone (Rasmusson et al. 2006). However, progesterone levels did not differ between groups and it was hypothesized that the conversion of progesterone to allopregnanolone may be impaired in women with PTSD. Although the HPA axis is known to play a key role as a regulator in the acute stress response, the potential modulatory effect of progesterone and allopregnanolone on functioning of the HPA axis has not previously been investigated in men and women with PTSD.

The present study examined the effects of sex and PTSD on ACTH, progesterone, and allopregnanolone responses to metyrapone in medically healthy men and follicular-phase premenopausal women with chronic PTSD and healthy controls. Since metyrapone blocks adrenal cortisol synthesis and inhibits the 11-β-hydroxysteroiddehydrogenase type-1 (11-β-HSD-1) enzyme, it results in the increase in ACTH. However, 11-β-HSD-1 is widely expressed in the central nervous system and inhibition of 11-β-HSD-1 increases other steroids that are synthesized proximal to the enzyme block by metyrapone. In line with this, administration of metyrapone has been shown to result in a robust rise of progesterone (Jahn et al. 2003). Since allopregnanolone is downstream from progesterone, we predicted that metyrapone would result in an increase in allopregnanolone as well. This is also supported by the findings that direct infusion of CRF and ACTH produces a potent increase in progesterone and allopregnanolone (Genazzani et al. 1998; Jahn et al. 2003).

Nocturnal blood sampling was obtained as a part of a three-night sleep study conducted in a General Clinical Research Center (GCRC). Nocturnal blood sampling provides the opportunity to evaluate the real-time association between acute HPA, progesterone, and allopregnanolone responses to metyrapone. We predicted that ACTH, progesterone, and allopregnanolone responses to metyrapone would be increased in PTSD subjects and in women. Further, given the possible feedback regulation of allopregnanolone and progesterone on HPA reactivity, we tested the hypothesis that the progesterone and allopregnanolone response would be associated with a decreased ACTH response and account for PTSD effects on the HPA response to metyrapone.

Methods

The present study used a cross-sectional, 2×2 design (PTSD/control×female/male) involving 85 medically healthy, non-medicated adults aged 19–39 years in an inpatient sleep laboratory. The study sample was comprised of 43 individuals with current chronic PTSD (49 % female) and 42 control subjects (50 % female). This sample was drawn from a larger study of 93 participants. Data from eight participants were excluded due to difficulties in blood collection over the night. Chronic PTSD was defined by fulfillment of DSM-IV criteria for chronic PTSD on the Clinician-Administered PTSD Scale (CAPS) and a CAPS score >40. Control subjects had no lifetime or current history of a PTSD diagnosis. Female participants were premenopausal (having at least one menstrual period in past 12 months) as determined by medical screening interview, and were scheduled during the follicular phase of the menstrual cycle. Exclusion criteria included history of traumatic brain injury; presence of neurologic disorders or systemic illness; use of psychiatric, anticonvulsant, antihypertensive, sympathomimetic, steroidal, statin or other prescription medications; obesity (defined as BMI >30); alcohol abuse or dependence in the prior 2 years; substance abuse or dependence in the previous year; any psychiatric disorder with psychotic features; bipolar disorder or obsessive compulsive

disorder; and pregnancy. Exclusion criteria for control subjects also included a lifetime history of major depressive disorder (MDD) or panic disorder. This research was approved by the Committee on Human Research at the University of California, San Francisco. All participants provided written informed consent before participating in any study procedures.

Measures

Psychiatric diagnoses and trauma history The CAPS was used to assess current and lifetime PTSD (Blake et al. 1995). The CAPS assesses the frequency and intensity of PTSD symptoms corresponding to the re-experiencing, avoidance, and hyperarousal symptoms described in the DSM-IV diagnostic criteria. Diagnosis of PTSD was based on symptoms experienced in the previous month, which were associated with the participant's self-identified worst traumatic event.

The Structured Clinical Interview for DSM-IV, Non-Patient edition (SCID-NP) was used to diagnose all other psychiatric disorders, including MDD (Spitzer et al. 1992). The type of trauma exposure and age of occurrence was assessed using the Life Stressor Checklist-Revised interview (Wolfe et al. 1996).

Metyrapone challenge and biological assays On the morning after the second night on the GCRC, subjects were given an oral dose of metyrapone of 750 mg every 4 h starting at 12 h before habitual sleep (HS) onset, for a total of three doses, and one dose of 2.5 g at HS along with 30 ccs of an antacid. Two hours before habitual sleep onset, a catheter was inserted in an antecubital vein for repeated sampling of blood on nights 2 and 3 (5.5 ccs every 15 min providing 32 samples for ACTH and progesterone (16 pre and 16 post metyrapone) and 20 samples for allopregnanolone (4 pre and 16 post metyrapone). Blood was sampled for up to 8 h following the final dose of metyrapone(ACTH: M=6.5 h; SD=1.9 h; progesterone: M=6.4, SD=1.9; allopregnanolone: M=6.7 h, SD=1.7 h).

Cortisol Blood was collected using an EDTA tube and plasma was separated by centrifugation. Plasma levels of cortisol were measured using the Access Chemiluminescent Immunoassay (Beckman Coulter, Fullerton, CA) at the Specialized Assay Core, Brigham and Women's Hospital.

ACTH Blood was collected using an EDTA tube and plasma was separated by centrifugation. Plasma levels of ACTH were measured using a commercially available immunoradiometric assay (DiaSorin Inc., Stillwater, Minnesota) at the Specialized Assay Core, Brigham and Women's Hospital. The DiaSorin ACTH immunoradiometric assay is designed to detect whole molecule ACTH and is a more sensitive method for the detection of ACTH in plasma than a radioimmunoassay.

Progesterone Blood was collected using a serum separator tube and serum was separated by centrifugation. Serum levels of progesterone were measured using a commercially available paramagnetic particle, chemiluminescent immunoassay (Beckman Coulter Inc, Fullerton, California) at the Specialized Assay Core, Brigham and Women's Hospital.

Allopregnanolone Blood was collected using an EDTA tube and plasma was separated by centrifugation. Concentrations of allopregnanolone were measured in ether-extracted plasma samples using a well-characterized 3H-radioimmunoassay as previously described (de Wit et al. 2001) conducted by the UCSD laboratory of Dr. Richard Hauger. The assay sensitivity is approximately 200 pg/ml, and the standard linear range is 0.2–50 ng/ml. Values were multiplied by 1.28 to correct for an extraction recovery of 78 %. Intra-assay coefficient of variation is approximately 5 %, while the inter-assay CV is approximately 8 %.

Statistical analysis

The four groups defined by gender and PTSD status were compared on demographic and clinical characteristics using F tests for continuous variables (or t tests when comparing only females and males with PTSD) and chi-squared tests for categorical variables. For analysis purposes, all hormone measurements were log-transformed because their distributions were right-skewed, and the change from pre- to post-metyrapone was exponential. Because subjects were measured until either waking or 8 h after lights out, whichever came first, and because of physical limitations that resulted in early discontinuation for some subjects, there was variability for each night in the number of measurements and the time of latest measurement, both within and between subjects. In this situation, using linear mixed effect models is preferred instead of traditional analytical approaches, such as area under the curve or repeated measures ANOVA because they allow for unbalanced groups, unequal number and timing of measurements, modeling time as continuous rather than a discrete set of points, and a wide variety of specifications available for modeling the covariance among the repeated measurements. Mixed models include both fixed and random effects. Fixed effects are for levels of a variable that are the only levels of interest (e.g., PTSD or gender). Random effects are for the levels of a variable that can be thought of as a sample drawn from some larger population of levels (e.g., the subjects in a study sample). Linear mixed models allow us to explicitly model individual hormone trajectories within each night via

subject-specific random effects. They also allow for prediction of subject-specific responses to metyrapone that can be used as outcome or predictor variables in further analyses.

Based on visual inspection of spaghetti plots of the subject-level serial hormone measurements at each night, and tests of the linearity of hormone level over the course of the night, we proceeded to analyze the association between hormone responses to metyrapone and the independent variables (PTSD status and gender) using linear mixed effect regression models. These models included fixed effects for PTSD status, gender, metyrapone status, time (15 min intervals) and their complete interaction, subject-specific random intercepts, and random slopes for pre- and post-metyrapone nights with an unstructured covariance structure, and the intra-measurement covariance was allowed to differ for each night. Model estimated means and differences of means (and 95 % confidence intervals) at lights-out were back-transformed to give the geometric mean or fold-change, respectively. In bivariate regression analyses, we also examined whether potential confounders (e.g., BMI, smoking, current major depression, history of childhood trauma (defined as two or more, versus one or no, categories of childhood trauma), and use of hormonal birth control) were associated with hormone responses to metyrapone.

Subject-specific responses to metyrapone were derived from the linear mixed models (i.e., the empirical best linear unbiased predictor of change from pre- to post-metyrapone) and used as mediator and outcome variables in a mediation analysis testing whether the progesterone and allopregnanolone responses mediated the relationship between PTSD status and the ACTH response. The 95 % confidence intervals for the direct and indirect effects were bootstrapped. F tests, t tests, X^2 tests, and linear mixed effects models were done with SAS version 9.3 (SAS Institute, Cary, NC). Simple mediation analyses were performed with Mplus (Muthén and Muthén 2011).

Results

Demographic data and clinical characteristics Sample characteristics are presented in Table 1. There were no significant differences in sex distribution between PTSD and control subjects, nor were there significant differences in age or education across all four groups. PTSD subjects were more likely to be of non-Caucasian race/ethnicity. Male PTSD subjects were more likely and female PTSD subjects were slightly less likely to be obese than controls. Male and female PTSD subjects did not differ in terms of CAPS scores, rates of current MDD, or history of childhood trauma (defined by the presence of two or more categories of childhood trauma as compared to one or none). Eleven control subjects reported a lifetime history of a traumatic criterion A1 event, but all had current CAPS scores of zero and none had a lifetime history of PTSD. As per the exclusion criteria, no control subjects met criteria for current MDD. Additionally, none of the control subjects reported a history of two or more categories of childhood trauma. There were no differences between PTSD and control women in use of hormonal birth control or current smoking of tobacco.

ACTH, progesterone, and allopregnanolone responses to metyrapone As expected, cortisol levels were significantly suppressed by metyrapone and there were no group or gender differences (pre-metyrapone geometric mean=28.2 ng/ml, 95 % CI [24.3, 32.5]) and post-metyrapone geometric mean=19.9 ng/ml 95 % CI [17.3, 22.9]). There were significant increases in ACTH, progesterone, and allopregnanolone from pre- to post-metyrapone in all of the groups (see Fig. 1 and Table 2). Linear mixed models indicated a significant group effect for ACTH such that PTSD participants had a 1.3-fold greater ACTH response compared to controls (95 % CI [1.1,1.54], $p<.05$) (Fig. 2). There was also a significant effect for gender such that females had a 2.04-fold greater ACTH response compared to men (95 % CI [1.72, 2.41], $p<.0001$) (Fig. 2). The group effect for ACTH in women was 1.39-fold greater than the group effect in men but the difference did not reach statistical significance (95 % CI [0.99, 1.95], $p=.056$).

Females had a 1.39-fold greater progesterone response than males (95 % CI [1.27, 1.53], $p<.0001$). We also found a significant group by sex interaction for progesterone (Fig. 2). Specifically, the difference between PTSD and controls was 1.48-fold greater in females compared to males (95 % CI [1.23, 1.78], $p<.05$), resulting from a larger progesterone response in PTSD (vs. controls) among women (1.23-fold difference, 95 % CI [1.07, 1.41], $p<.001$) compared to a smaller progesterone response in PTSD (vs. controls) in men (0.83-fold difference, 95 % CI [0.74, 0.94], $p<.001$). There was also a significant sex difference in allopregnanolone responses, such that women had a 1.33-fold greater allopregnanolone response compared to men (95 % CI [1.12, 1.57], $p<.001$). There were no significant group differences for PTSD status and no group by sex interaction for allopregnanolone. Excluding the seven women (two controls, five PTSD+; Table 1) taking oral contraceptives, all of the findings regarding the ACTH, progesterone, and allopregnanolone responses to metyrapone were very similar, except that the slight trend of a PTSD by gender by metyrapone interaction found previously for ACTH became even less statistically significant ($p=.1033$).

Potential confounders There were no significant associations between BMI, current smoking status, current MDD, childhood trauma (defined as two or more, versus one or no, categories of childhood trauma), and use of hormonal birth

Table 1 Demographic and clinical characteristics by gender and PTSD status

	Males		Females			
Variable	Control (*N*=21)	PTSD+(*N*=22)	Control (*N*=21)	PTSD+(*N*=21)	Total (*N*=85)	*p* value
Age (mean±SD)	30.5±8.86	30.9±6.45	30.4±7.83	30.4±6.95	30.6±7.43	0.99[a]
Education						
Some HS/HS Grad	2 (9.5 %)	5 (22.7 %)	1 (4.8 %)	2 (9.5 %)	10 (11.8 %)	0.68[b]
Some College/College Grad	15 (71.4 %)	14 (63.6 %)	14 (66.7 %)	15 (71.4 %)	58 (68.2 %)	
Grad/Prof School	4 (19.0 %)	3 (13.6 %)	5 (23.8 %)	4 (19.0 %)	16 (18.8 %)	
Race/ethnicity						
African American	1 (4.8 %)	3 (13.6 %)	0 (0.0 %)	2 (9.5 %)	6 (7.1 %)	0.048[b]
Asian/Hawaiian/Pacific Islander	4 (19.0 %)	4 (18.2 %)	3 (14.3 %)	2 (9.5 %)	13 (15.3 %)	
Caucasian	16 (76.2 %)	10 (45.5 %)	15 (71.4 %)	13 (61.9 %)	54 (63.5 %)	
Hispanic	0 (0.0 %)	5 (22.7 %)	3 (14.3 %)	1 (4.8 %)	9 (10.6 %)	
Other[c]	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	3 (14.3 %)	3 (3.5 %)	
Current CAPS score[d] (mean±SD)	0.0±0.0	51.9±13	0.0±0.0	56.2±16.7	54±14.9	0.34[a]
Current MDD[e]	0 (0.0 %)	5 (22.7 %)	0 (0.0 %)	2 (9.5 %)	7 (8.2 %)	0.019[b]
Childhood trauma ≤14 years of age[f]	0 (0.0 %)	9 (40.9 %)	0 (0.0 %)	13 (61.9 %)	25 (29.4 %)	0.12[b,g]
Hormonal birth control	NA	NA	2 (9.5 %)	5 (23.8 %)	7 (8.2 %)	0.21[b]
BMI						
Underweight ≤18.5	2 (9.5 %)	0 (0.0 %)	0 (0.0 %)	0 (0.0 %)	2 (2.4 %)	<.0001[b]
Normal weight=18.5–24.9	9 (42.9 %)	4 (18.2 %)	12 (57.1 %)	13 (61.9 %)	38 (44.7 %)	
Overweight=25–29.9	10 (47.6 %)	5 (22.7 %)	6 (28.6 %)	6 (28.6 %)	27 (31.8 %)	
Obesity=BMI of 30+	0 (0.0 %)	13 (59.1 %)	3 (14.3 %)	2 (9.5 %)	18 (21.2 %)	
Smoker[h]	3 (14.3 %)	4 (18.2 %)	5 (23.8 %)	7 (33.3 %)	19 (22.4 %)	0.48 [b]

[a] Based on *F* test

[b] Based on Chi-square test

[c] These three subjects endorsed Hispanic ethnicity but did not select a racial descriptor. Seven additional subjects endorsed Hispanic ethnicity, in addition to a racial category of Caucasian or African-American race yielding a total of 10 subjects self-identifying as Hispanic in this sample

[d] Control subjects had CAPS scores of zero or had an absence of criterion A events. Comparison of male and female PTSD subjects on current CAPS score, $p=0.81$

[e] Absence of current MDD was required for inclusion into the control group. Comparison of male and female PTSD subjects on rate of current MDD, $p=0.24$

[f] Childhood trauma exposure was defined, based on findings from our prior research, by exposure to two or more categories of childhood trauma. Three (7.1 %) control subjects reported a history of one category of childhood trauma

[g] Chi-square test compared frequency of childhood trauma between male and female PTSD subjects only

[h] Based on diary

control with ACTH, progesterone, and allopregnanolone responses in bivariate analysis; therefore, these variables were not included in further analyses.

Mediation of the relationship between PTSD on ACTH by progesterone and allopregnanolone Mediation analyses were performed to examine whether progesterone or allopregnanolone mediated the relationship between PTSD and ACTH (see Figs. 3 and 4). The progesterone, allopregnanolone, and ACTH variables differ from those used above, since these were subject-specific predicted values derived from the linear mixed models and include the increase of the rate of change of hormone levels from pre- to post-metyrapone. Separate analyses were carried out for men and women (Figs. 3 and 4).

Contrary to our hypothesis, progesterone was strongly positively associated with ACTH, while controlling for PTSD, among both women and men (fold-increase in ACTH per 2.72-fold increase in progesterone: among women=4.06, 95 % CI [2.79, 5.9], $p<.001$; among men=2.62, 95 % CI [1.31, 5.24], $p<.001$). The effect of PTSD on ACTH was also positive with female and male PTSD subjects experiencing a 1.52-fold and 1.10-fold greater ACTH response, respectively, compared to controls. After adjusting for progesterone, the (unmediated direct) effect of PTSD on ACTH women was attenuated in women (fold difference for

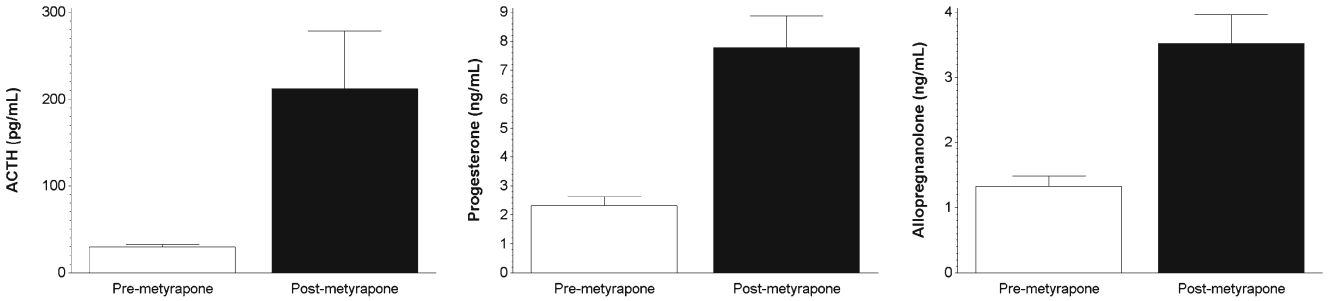

Fig. 1 Mean levels (and upper 95 % CI) of ACTH, progesterone, and allopregnanolone at pre- and post-metyrapone. The means shown were calculated at the time point that corresponded with the time at which the sample mean of ACTH reached its maximum (6.5 h after lights out)

PTSD vs. control=1.15, 95 % CI [1.1, 1.22], $p<.001$), whereas it grew stronger in men (fold difference for PTSD vs. control=1.31, 95 % CI [1.22, 1.44], $p<.001$). The direct effect between PTSD and progesterone was positive in women (fold-difference for PTSD vs. control=1.22, 95 % CI [1.22, 1.23], $p<.001$), but it was negative among men (fold-difference for PTSD vs. control=0.84, 95 % CI [0.83, 0.84], $p<.001$). Finally, progesterone was found to mediate the relationship between PTSD and ACTH, in both women and men, as indicated by significant indirect effects of PTSD via progesterone, but the mediation differed by gender due to the positive PTSD effect on progesterone among women and the negative PTSD effect on progesterone among men. The mediation of PTSD on ACTH by progesterone was only partial in both men and women since the unmediated direct effects of PTSD on ACTH remained significant.

Mediational analyses were also performed to examine whether allopregnanolone mediated the relationship between PTSD and ACTH (see Fig. 4). Contrary to our hypothesis, allopregnanolone was strongly positively associated with ACTH while controlling for PTSD (fold-increase in ACTH per 2.72-fold increase in allopregnanolone among women=2.36, 95 % CI [2.04, 3.44], $p<.001$; and among men=2.26, 95 % CI [1.38, 2.34], $p<.001$). The effect of PTSD on ACTH was positive with female PTSD subjects experiencing a 1.52-fold greater ACTH response compared to controls (95 % CI [1.52, 1.53], $p<.001$) and male PTSD subjects experiencing a 1.10-fold greater ACTH response compared to controls (95 % CI [1.10, 1.11], $p<.001$). After adjusting for allopregnanolone, the (unmediated direct) effect of PTSD was stronger (fold difference for PTSD vs. control among women=1.6, 95 % CI [1.6, 1.65], $p<.001$; among men=1.27, 95 % CI [1.16, 1.28], $p<.001$). There was also a significant direct effect between PTSD and allopregnanolone (fold-difference for PTSD vs. control among women=0.94, 95 % CI [0.93, 0.95], $p<.001$; and among men=0.84, 95 % CI [0.84, 0.85], $p<.001$). Finally, allopregnanolone was found to mediate the relationship between PTSD and ACTH as indicated by a significant indirect effect of allopregnanolone (fold difference for PTSD vs. control among women=0.95, 95 % CI [0.93, 0.96], $p<.001$; and among men=0.87, 95 % CI [0.87, 0.94], $p<.001$), and this mediation was partial since the unmediated direct effect of PTSD on ACTH remained significant. Though the negative indirect effect of PTSD on ACTH via allopregnanolone is slightly stronger (closer to 0) in men than women, it is not statistically significantly different from the indirect effect observed in women. The unmediated direct effect of PTSD on ACTH is larger in magnitude than the total PTSD effect because the partial mediation via allopregnanolone is negative (i.e., PTSD subjects tended to have lower allopregnanolone responses than controls and this dampened allopregnanolone response was associated with a greater ACTH response).

Discussion

The present study found that the ACTH response to metyrapone was higher in PTSD subjects compared to controls and in women compared to men in a study that sampled plasma immediately and at intervals over 8 h. Our finding of a greater ACTH response associated with PTSD is similar to other research studies that also examined responses during the initial peak and at intervals over several hours after drug administration (Golier et al. 2009; Yehuda et al. 1996), but differ from our previous work in which blood levels of ACTH were determined at a single time point, delayed 8–9.5 h after an overnight metyrapone test (Neylan et al. 2003; Otte et al. 2007). Metyrapone blocks cortisol synthesis for approximately 4 h; therefore, the timing of measurement of ACTH is critical in interpreting findings across studies. Initially, metyrapone increases ACTH by removing negative feedback when cortisol production is blocked. Over time, the resumption of cortisol production results in a consequent decrease in ACTH through negative feedback of cortisol on hypothalamic and pituitary glucocorticoid receptors. Our findings of increased ACTH to metyrapone in PTSD may reflect heightened hypothalamic CRF hypersecretion (Lisansky et al. 1989;

Table 2 ACTH, progesterone, and allopregnanolone responses to metyrapone by PTSD status within gender

Hormone measure	Time point	Women			Men		
		PTSD Estimate (95 % CI)	Control Estimate (95 % CI)	PTSD vs. control Between group fold-difference (95 % CI)	PTSD Estimate (95 % CI)[a]	Control Estimate (95 % CI)	PTSD vs. control Between group fold-difference (95 % CI) [a]
ACTH (ng/ml)	Pre-metyrapone[a]	0.01 (0.01–0.01)*	0.01 (0.01–0.02)*	0.91 (0.66–1.25)	0.01 (0.01–0.02)*	0.01 (0.01–0.02)*	1 (0.72–1.38)
	Post-metyrapone[a]	0.05 (0.04–0.07)*	0.04 (0.03–0.05)*	1.39 (0.95–2.02)	0.02 (0.02–0.03)*	0.02 (0.02–0.03)*	1.1 (0.77–1.58)
	Post vs. Pre fold-change[a]	4.85 (4.05–5.81)*	3.16 (2.66–3.76)*	1.53 (1.19–1.97)**	2.02 (1.71–2.39)*	1.57–2.14)*	1.1 (0.88–1.38)
Progesterone (ng/ml)	Pre-metyrapone[a]	1.26 (1.02–1.57)***	1.41 (1.14–1.74)***	0.9 (0.66–1.22)	1.1 (0.89–1.37)	0.95 (0.76–1.17)	1.17 (0.86–1.58)
	Post-metyrapone[a]	3.66 (2.92–4.58)*	3.31 (2.67–4.1)*	1.11 (0.81–1.51)	1.89 (1.52–2.34)*	1.94 (1.57–2.41)*	0.97 (0.72–1.32)
	Post vs. Pre fold-change[a]	2.89 (2.63–3.19)*	2.35 (2.14–2.59)*	1.23 (1.07–1.41)***	1.71 (1.56–1.87)*	2.05 (1.89–2.23)*	0.83 (0.74–0.94)***
Allopregnanolone (ng/ml)	Pre-metyrapone[a]	0.89 (0.67–1.18)	0.93 (0.72–1.2)	0.96 (0.66–1.4)	1.05 (0.78–1.42)	0.86 (0.66–1.12)	1.22 (0.82–1.81)
	Post-metyrapone[a]	1.88 (1.47–2.41)*	2.1 (1.67–2.62)*	0.9 (0.64–1.25)	1.59 (1.24–2.04)**	1.54 (1.23–1.93)**	1.03 (0.73–1.44)
	Post vs. Pre fold-change[a]	2.11 (1.78–2.49)*	2.26 (1.92–2.65)*	0.93 (0.74–1.18)	1.51 (1.26–1.81)*	1.79 (1.54–2.08)*	0.84 (0.66–1.07)

[a] Geometric means and fold-changes were estimated from linear mixed models of log of hormone measure with fixed effects for group, gender, metyrapone status (pre and post), time, and the complete interaction of these four variables; random effects included a subject-specific intercept and random slopes (different ones for the pre- and post-metyrapone nights), and a different intra-measurement covariance for each night

*$p<.0001$; **$p<.001$; ***$p<.05$

Young et al. 1994), consistent with previous findings of elevated CSF corticotrophin-releasing hormone in PTSD (Baker et al. 2005; Bremner et al. 1997; Sautter et al. 2003) and evidence of a link between single nucleotide polymorphisms of the corticotropin releasing hormone type 1 receptor (CRHR1) gene with PTSD (Amstadter et al. 2011).

Studies have also found enhanced glucocorticoid receptor sensitivity in PTSD subjects (Grossman et al. 2003; Newport et al. 2004; Stein et al. 1997; Yehuda et al. 2002, 2004). The contrasting findings of a lower ACTH response to metyrapone in PTSD in our earlier studies using delayed ACTH measurement (Neylan et al. 2003; Otte et al. 2007) can be explained by potential receptor down-regulation or enhanced negative feedback in the face of increased CRF drive. Eventually, as cortisol production recovers and levels increase in the hours after the metyrapone challenge, the greater glucocorticoid receptor sensitivity in PTSD subjects may result in enhanced feedback resulting in lower ACTH concentrations.

We also found a robust gender difference in the ACTH response to metyrapone, indicating increased HPA axis activity in women, and differences in progesterone and allopregnanolone, putative modulators of HPA function. Much of our understanding of the neurobiological processes in PTSD is derived from research that was conducted in males or from studies that failed to examine the impact of sex. Our findings suggest that neurobiological mechanisms central to the acute stress response involved in PTSD may differ in men and women.

Our findings of elevated HPA axis reactivity may reflect increased CRF drive in women. Although there are some inconsistencies (Sterrenburg et al. 2011, 2012), there is evidence from preclinical research suggesting that female rodents have greater CRF expression in the paraventricular nucleus of the hypothalamus (Desbonnet et al. 2008; Duncko et al. 2001; Iwasaki-Sekino et al. 2009; Viau et al. 2005). Studies have shown that female rodents had greater ligand binding to CRF_1 receptors in the amygdala after puberty (Weathington and Cooke 2012), greater Gs-coupled CRF_1 receptor signaling, and impaired β-arrestin2 recruitment by brain CRF_1 receptors, which may confer excessive stress sensitivity and responsivity (Bangasser and Valentino 2012). Some studies have also suggested slower glucocorticoid negative feedback inhibition in women than men (Kudielka and Kirschbaum 2005; Gallucci et al. 1993; Heuser et al. 1994). Rodent studies have shown reduced MR and GR number (Turner 1990) and impaired translocation following chronic adolescent stress in females, which may contribute to impaired or slower glucocorticoid feedback (Bourke et al. 2013). Our finding of heightened ACTH responses to metyrapone in women, an effect previously linked with PTSD, suggests that regulation of the

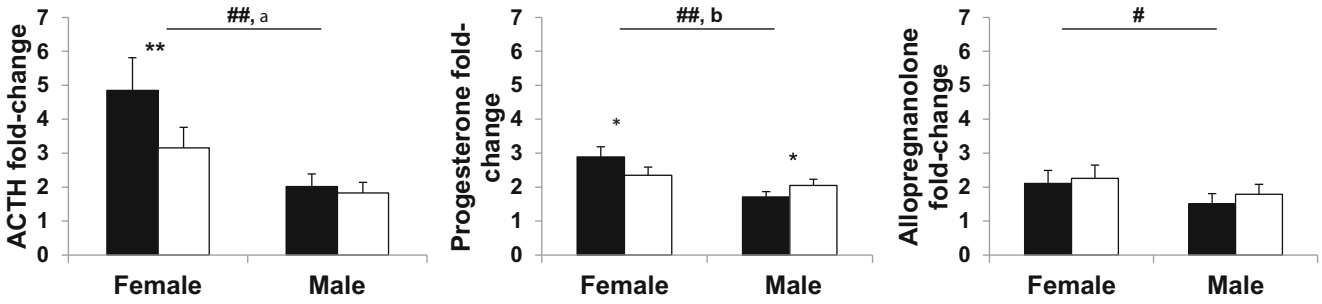

Fig. 2 ACTH, progesterone, and allopregnanolone responses to metyrapone by gender and PTSD status. Figure shows the estimated fold-change in mean hormone level in response to metyrapone. *Error bars* show upper 95 % confidence interval. Within-gender comparisons of hormone responses for PTSD vs. controls, *$p<.05$, **$p<.001$. Comparison of hormone responses in females vs. males, #$p<.001$, ##$p<.0001$. [a]ACTH response for PTSD vs. control, $p<.05$. [b]Group by gender interaction for progesterone response, $p<.0001$

HPA axis may be a potential mechanism involved in the heightened risk for PTSD in women.

Several sex-specific neuroendocrine mechanisms may mediate our findings. Because progesterone secretion is stimulated by ACTH, the large increases in ACTH secretion resulting from metyrapone treatment provide strong stimulatory drive to release progesterone and allopregnanolone from the adrenal cortex. The sensitivity to glucocorticoid negative

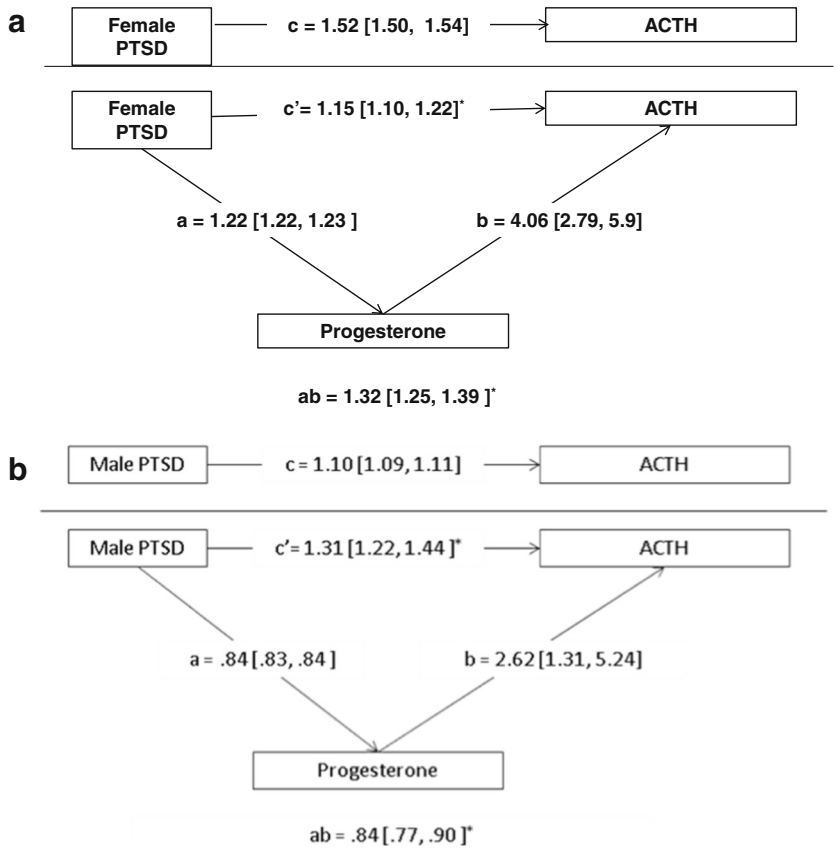

Fig. 3 Mediation model showing that the effect of PTSD (independent variable) on ACTH response (outcome) is partially mediated by progesterone response (mediator) and that the direction of mediation differs by gender. **a** Mediation among women. **b** Mediation among men. The *top path diagram* in each figure shows the total effect (*c*) between the independent variable (PTSD) and the outcome (ACTH response). The *lower diagram* in each figure shows the test of whether some portion of the total effect of PTSD is accounted for by the indirect effect (cross-product of unstandardized regression coefficients *a* and *b*) of the mediator (progesterone response), and the remaining unmediated direct effect (*c*′) of PTSD on ACTH responses. In this case, the mediation is only partial since both the indirect effects and direct effects remain statistically significant. Outcome and mediator variables were in log-scale and the regression coefficients and corresponding 95 % confidence intervals were back-transformed to give the fold-effect of a one-unit change in the independent variable. For example, without the mediator, female PTSD subjects (**a** top path diagram) have 1.52-fold greater ACTH response than controls. All estimates were statistically significant with $p<.01$. *Bias corrected bootstrapped confidence intervals

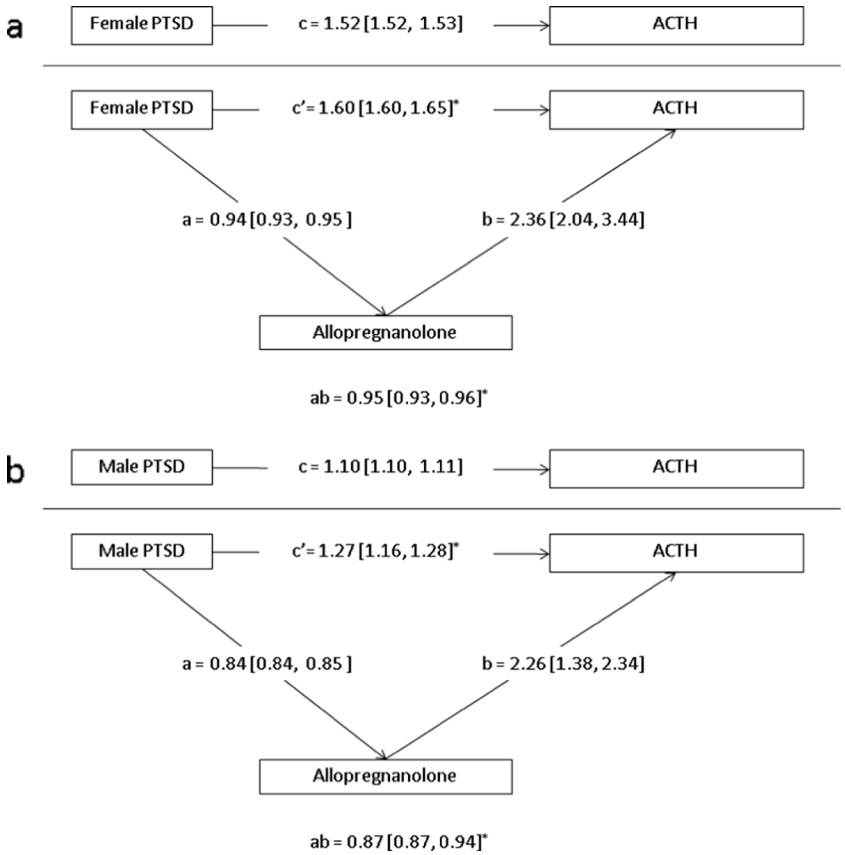

Fig. 4 Mediation model showing that the effect of PTSD (independent variable) on ACTH response (outcome) is partially mediated by allopregnanolone response (mediator) by gender. **a** Mediation among women. **b** Mediation among men. The *top path diagram* in each figure shows the total effect (*c*) between the independent variable (PTSD) and the outcome (ACTH response). The *lower diagram* in each figure shows the test of whether some portion of the total effect of PTSD is accounted for by the indirect effect (cross-product of unstandardized regression coefficients *a* and *b*) of the mediator (allopregnanolone response), and the remaining unmediated direct effect (*c*′) of PTSD on ACTH responses. In this case, the mediation is only partial since both the indirect effects and direct effects remain statistically significant. Outcome and mediator variables were in log-scale and the regression coefficients and corresponding 95 % confidence intervals were back-transformed to give the fold-effect of a one-unit change in the independent variable, i.e., values <1 correspond with decreases and values >1 correspond with increases. For example, without the mediator, female PTSD subjects (**a** top path diagram) have 1.52-fold greater ACTH response than controls. All estimates were statistically significant with $p<.001$. *Bias corrected bootstrapped confidence intervals

feedback, however, differs between men and women (Bangasser and Valentino 2012). In agreement with these observations, we found greater ACTH responses to metyrapone in women compared to men, especially in the PTSD groups (Fig. 2). Furthermore, androgens and estrogens exert differential regulatory actions on hormone secretion by the adrenal cortex (Kitay 1965), anterior pituitary (Coyne and Kitay 1969, 1971; Viau and Meaney 2004), and hypothalamus (Handa et al. 1994; Viau and Meaney 1996; Viau et al. 2001), which may also have contributed to our observed effects of metyrapone treatment on progesterone and allopregnanolone levels in female vs. male subjects.

We have also found that metyrapone treatment stimulated greater increases in plasma levels of progesterone and allopregnanolone in women compared with the largest differential progesterone response occurring between female and male PTSD subjects (Fig. 2). In women, both the adrenal cortex and ovaries can synthesize progesterone. Furthermore, human corpus luteal cells express 5α/5β-reductases and 3α-hydroxy-steroid oxidoreductase required for steroidogenic synthesis of allopregnanolone. Accordingly, the human corpus luteum has been shown to synthesize and secrete allopregnanolone (Ottander et al. 2005). Consistent with these in vitro findings, circulating levels of allopregnanolone have been found to increase twofold in women from the follicular to the luteal phase of the menstrual cycle (Ottander et al. 2005). Although the testis can convert progesterone to allopregnanolone (Trbovich et al. 2004), the corpus luteum appears to produce considerably higher levels of allopregnanolone. This sexual difference may contribute to our data showing greater allopregnanolone responses to metyrapone in women compared to men.

With regard to the effect of metyrapone on the pituitary–gonadal axis, glucocorticoids have been reported to exert a negative feedback regulation of hypothalamic GnRH release

and gonadotropin secretion in women. When normally cycling women are treated with a hydrocortisone dose to produce a fourfold increase in urinary free cortisol concentration, the LH interpulse interval becomes prolonged and serum levels of LH, FSH, and progesterone decrease consistent with glucocorticoid negative feedback on the pituitary–gonadal axis (Saketos et al. 1993). Hydrocortisone treatment, however, did not change serum estradiol levels in this study. Glucocorticoids have also been found to directly inhibit testicular and ovarian steroidogenesis. However, the sensitivity of the pituitary–gonadal axis to glucocorticoid actions may differ between women and men. The effect of metyrapone on adrenocortical and ovarian hormone levels has been studied in women with polycystic ovary syndrome (PCOS), which is an endocrine disorder associated with excess androgen secretion. After metyrapone (30 mg/kg PO) was administered at midnight, plasma levels measured at 8–9 a.m. the next day were significantly increased from the pre-metyrapone baseline for androstenedione (1.72 to 3.6 ng/ml) and 17-hydroxyprogesterone (0.7 to 3.4 ng/ml) while plasma estradiol decreased (54.7 to 5.8 pg/ml) and plasma testosterone did not change (0.35 to 0.30 ng/ml) (Loughlin et al. 1986). Although the effect of metyrapone-induced cortisol hyposecretion on circulating levels of LH and FSH has not been studied, the published data suggest that metyrapone may inhibit or have no effect on gonadal hormone secretion.

In our meditational model, we found that PTSD subjects tended to have lower increases in plasma allopregnanolone responses to metyrapone. This finding is consistent with the previous work by Rasmusson et al. that found lower CSF concentrations in women with PTSD compared to controls (Rasmusson et al. 2006). Our study replicates these findings and demonstrates an effect in both women and men. While progesterone and allopregnanolone are present in women and men, there may be gender differences in their synthesis or in $GABA_A$ receptor sensitivity to these neurosteroids (Pinna et al. 2004), although we did not find a sex by allopregnanolone interaction on ACTH. Rasmusson speculated that reductions in allopregnanolone could result from decreased conversion of 5α-DHP to allopregnanolone. Specifically, 3α-hydroxysteroid dehydrogenase (3α-HSD) is the enzyme that converts 5α-DHP to allopregnanolone, can also convert allopregnanolone to 5α-DHP, reducing allopregnanolone levels (Penning et al. 2004). This may occur as a result of stress that can increase the accumulation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), resulting in impaired inhibition of 3α-HSD oxidase activity (Rasmusson et al. 2006). 3α-HSD actions may also be influenced by 3α-HSD gene polymorphisms, mutations, or dysregulation of gene expression (Hou et al. 1998).

We predicted that the progesterone and allopregnanolone response would reduce the ACTH response due to feedback of progesterone and allopregnanolone on HPA reactivity and account for the effects of PTSD on the HPA response to metyrapone. Contrary to our initial prediction of an inverse relationship, progesterone and allopregnanolone were positively associated with ACTH. This is consistent with studies that have found that progesterone and allopregnanolone increase following injections of CRF and ACTH (Torres et al. 2001) and in response to various stressors, including swim stress (Purdy et al. 1991), foot shock (Barbaccia et al. 1997), and CO_2 exposure (Barbaccia et al. 1996). Yet, the function of increased progesterone and allopregnanolone may be to counter-regulate increasing ACTH. Progesterone has multiple actions at regulatory sites throughout the HPA axis, which may account for the association with ACTH seen in our study. Progesterone modulates CRF promoter activity through cyclic AMP (cAMP) response elements (Ni et al. 2004). Progesterone can also directly bind to type I and type II glucocorticoid receptors and interfere with glucocorticoid negative feedback regulation (Turner 1997; Xu et al. 1990). Progesterone may also augment the HPA axis response by increasing production of chaperone proteins (Wochnik et al. 2005). Additionally, progesterone may have indirect effects through allopregnanolone, which can modulate the HPA axis via GABA. Rodent studies have shown that inhibition of GABA reduces ACTH and corticosterone secretion (Jones et al. 1984). Allopregnanolone administration blunts ACTH and corticosterone responses to stress (Patchev et al. 1996), decreases CRF-enhanced startle (Toufexis et al. 2004), and counteracts anxiogenic responses to CRF during challenging maze tasks (Patchev et al. 1994, 1996). It is possible that progesterone, allopregnanolone, and ACTH initially increase together acutely, but that the inhibitory effect only becomes apparent after a delay. It is also possible that allopregnanolone is ineffective at blocking the HPA axis activity in the context of elevated CRF, as has been suggested by research examining the HPA effects of the pregnane steroid alfaxolone (Britton et al. 1992).

Our results suggested a partial mediation of the relationship between PTSD and ACTH by progesterone in both men and women as well as a direct effect of PTSD on ACTH. There were differences in the direction of the relationship between PTSD and progesterone in men and women, such that there was a positive PTSD effect on progesterone among women and a negative PTSD effect on progesterone among men. We also found partial support for a meditational role for allopregnanolone; the allopregnanolone response partially mediated the relationship between PTSD and ACTH, but there remained a direct effect of PTSD on ACTH.

Our findings add evidence to the growing literature on sex differences in the neurobiological mechanisms that may account for the greater susceptibility to mood and anxiety disorders in women. These findings highlight the importance of considering the influence of gender in biological studies of PTSD. The development of pharmacological agents that target

CRF drive such as CRH R1 antagonists or allopregnanolone (e.g., synthetic neurosteroids) is an important avenue for scientific exploration and may advance available treatment options for symptomatic survivors of trauma and especially women (Adamec et al. 2010; Reddy 2010). Further characterizing sex differences in these processes will advance our understanding of the pathophysiology of PTSD, and may ultimately lead to better-targeted, more efficient and effective treatment for PTSD in both men and women.

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