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Differences in the Central Neural Activation under Emotional Stress across the Menstrual Cycle.

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by

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Abstract.

The present study had several goals. First, we aimed to investigate the potential differences in the activation of the corticolimbic structures during emotional stress in healthy women across the menstrual cycle using stress imagery. Second, we searched for differences in the subjective anxiety under emotional stress across the menstrual cycle and tried to correlate the perceived level of anxiety to activation of the specific corticolimbic structures. Third, we attempted to compare central neural activation of women in follicular and in luteal phases of the menstrual cycle separately to that of men during emotional stress to investigate potential differences in neural response. We used perfusion based functional magnetic resonance imaging (MRI) and blood oxygen level dependent (BOLD) contrast to measure cerebral blood flow response to the emotional stress using stress imagery in 29 healthy volunteers (9 women in follicular phase, 10 women in luteal phase, and 10 men). Cycle-dependent comparison of the stress response in women revealed that women in the follicular phase had greater activation in the areas of the ventro-medial prefrontal cortex (VMPFC), with levels of activation comparable to those of men, and anterior insula, while women in the luteal phase of their menstrual cycle demonstrated increase blood flow in the areas of the anterior cingulate and hippocampus at $P = 0.01$. Males showed overall greater degree of corticolimbic activation, specifically in the bilateral hippocampi and right prefrontal cortex regardless of which group of women they were compared to. When compared to women in different phases of the menstrual cycle specifically, men showed greater cerebral blood flow in bilateral cingulate cortices and right hippocampus compared to women in the follicular phase, and

bilateral striatum, amygdala, bilateral hippocampi when compared to women in the luteal phase. We did not observe different levels of self-reported anxiety during stress imagery across the menstrual cycle, however, women in their luteal phase showed a positive correlation of the self-reported anxiety levels and cerebral blood flow in the posterior insula at the threshold level of $P = 0.05$. The results of our study are consistent with the previously available information regarding the differences in the corticolimbic activation across the menstrual cycle in women and in women vs. men. In addition to that, our data supports the correlation of the levels of anxiety and insular activation in the luteal phase of the menstrual cycle and could represent an initial step in uncovering the mechanisms regulating stress response, anxiety and their relation to the hormonal status.

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Introduction.

Stress has long been known to have various effects on lives. Through numerous studies it has been associated with depression, impairment in cognitive function, weakened immune system response and an earlier onset of age-related disease [1-5]. Recently, interaction of gender and stress reactivity have been proposed as a potentially important influence on prevalence of various health problems in men and women, in addition to sociologic, developmental and cultural factors [6, 7]. While there is a definitive pattern in prevalence of several physical and psychiatric disorders depending on gender [7-10], some of those differences become more prominent during women's reproductive years, and gradually decline after menopause, suggesting that the gender-specific pattern of observed prevalence of diseases may be partly due to the effects of sex hormones [11].

Although the exact neural circuitry of psychological stress remains unclear, considerable progress has been made in the uncovering of cortical and subcortical structures that regulate stress response, reporting activations in the prefrontal cortex, anterior and posterior cingulate, caudate, putamen, hippocampus, amygdala and insula [12, 13]. In emotion and depression research, same regions have been reported to be involved in emotional information processing [14-16], and to show altered activity in the depressed state [17, 18]. Secondary to the evidence of variation in neuroendocrine stress response depending on gender [7, 9], some investigators have also approached the idea of difference in neural circuit's response in males and

females, reporting unequal activation of brain structures during psychological stress as well as during emotion processing, with changes occurring in the corticolimbic and striatal regions that are important in emotional and stress processing [19, 20].

While more imaging evidence is becoming available regarding the activation of neural circuits under the influence of stress depending on gender, and proven variation in the neuroendocrine stress response in women depending on their hormonal status [21-23], few studies have compared brain activation at different points in the hormonal cycle. Those that are available tend to focus on cognitive tasks such as mental rotation and word-stem-completion tasks [24-26], reporting differences in the size and localization of active areas. The only study to compare psychological stress at different points of menstrual cycle was Protopopescu et all, 2005, using words with negative, positive and neutral context as stimuli, and reporting differences in the activation of orbitofrontal cortex (OFC), cingulate and insula – regions important in the emotional and stress response [27]. These results begin to shed light on the question of brain response to the psychological stress across menstrual cycle, yet the differences in the brain response to the emotionally stressful stimuli are still much unidentified.

To address these questions, we combined functional magnetic resonance imaging (fMRI) with investigation of the emotional stress response in females at two different phases of the menstrual cycle. Functional MRI blood oxygen level detection (BOLD) was used as a neuroimaging technique due to its optimal contrast for brain

activation mapping and consistent reproducibility [28, 29]. Three groups, 2 groups of females and 1 group of male volunteers participated in the study. All the participants were scanned during exposure to two individually developed scripts trials of stress and two neutral non-stress situations presented in random order. Based on the previous studies we expected to see a different response and activation patter during stress trials in structures previously reported to be involved in emotional processing, e.g. hippocampus, amygdala, anterior and posterior cingulate, prefrontal cortex and insula in women depending on the stage of their menstrual cycle. We also expected differences in the activation of women in different phases of the menstrual cycle when compared to men during stress imagery. Lastly, we were looking to investigate the potential differences in the levels of self-reported anxiety during emotional stress in healthy women across the menstrual cycle, and a possible correlation of the levels of perceived anxiety and cortical blood flow the specific corticolimbic structures.

Methods.

Subjects. Between May 2004 and August 2007 21 healthy, regularly cycling women (menstrual cycle length 25-31), reportedly not on birth control (mean age = 31.0, SD= 8.6) enrolled in the study after given written informed consent. Two participants were excluded from the study. One of the participants was excluded due to the fact that she reported being on oral contraceptive at the time of the study after, the fMRI was completed. Second participant was excluded from analysis secondary to the inability to establish the day and phase of her menstrual cycle at the time of fMRI. Nineteen women (mean age = 31.6, SD = 9.0) successfully completed the study and were included in the analysis.

Ten of the females included in the analysis were scanned during their luteal phase (menstrual cycle days 17-27), and 9 females were scanned during their follicular phases (menstrual cycle days 1-13). I have participated in that process, guiding the participant through scans and recording their physiological data throughout the imaging procedure.

Also, ten men who were scanned previously (mean age = 28.9, SD = 10.1), were chosen to closely resemble both groups of female participants by age, education, and race (table 1). All participants completed demographic assessment during initial visit. All subjects were recruited through public advertisement in the area of New Haven, Connecticut. All procedures were approved by the Human Investigation Committee of the Yale University School of Medicine.

Groups	Education		Race		
	t	p	ChiSquare	DF	P
HM vs. HF	0.68	0.507	1.444	3	0.6952
HM vs. HL	-1.22	0.2425	4.8	3	0.1870

Table 1. Comparison of the groups by education and race.

Hormonal assessment.

All women underwent hormonal assessment to confirm the phase of their

menstrual cycle. During the initial visit all women filled out the questionnaire where

they reported the length of their menstrual cycle and the regularity. During a different

visit all women underwent a blood draw to verify the expected levels of estrogen and

progesterone relative to their self-report of where they were expected to be in their

cycle. On the day of fMRI they were asked to report the day of their menstrual cycle

again. All the menstrual cycle information was confirmed and verified by me using

the length and day of the menstrual cycle reported during the first visit for the

calculation of the expected day to ensure the correct phase on the date of the fMRI

and date of the laboratory assessment. Hormonal assays were conducted by Yale

Laboratory for Surgery, Obstetrics and Gynecology (LSOG).

Imagery script development session.

In the week prior to the fMRI session, scripts for the guided imagery induction

were developed in a structured clinical interview session. Three *stress imagery scripts*

were developed for each subject. They were based on the subjects' description and

ratings of three separate personal, stressful events that were experienced as the "most stressful" within the past year. "Most stressful" was determined by having each subject rate their level of emotional distress experienced on a 10-point Likert scale, where "1=not at all stressful" and "10=the most distress they felt recently in their life". Only situations rated as 8 or above on this scale were accepted as appropriate for script development. Examples of stressful situations included breakup with significant other, a verbal argument with a family member, or unemployment-related incident. In addition to the stress scripts, three *neutral-relaxing scripts* were also developed from personalized, neutral situations. Examples of neutral relaxing situations included a day at the park, a summer beach scene, or relaxing Sunday afternoon reading.

A "script" or description of each stress and neutral situation was developed using Scene Development Questionnaire (SDQ) and methods described and used previously [30-34]. Briefly, the SDQ method obtains specific details on the physical, interpersonal, verbal/cognitive context, and bodily responses experienced for each situation. On the basis of these detail, two stress and two neutral scripts of 2 min in length were developed for each subject and recorded on an audiotape for the fMRI session. The order of the stress and neutral scripts was assigned randomly. Subjects remained blind to the order of the imagery condition until imagery induction on each day.

Imagery training session.

To reduce variability in imagery ability and to train all subjects in progressive relaxation, a one-session progressive relaxation and guided imagery training session was conducted with all subjects according to procedures outlined by Miller et. al. 1987 and used previously in imagery studies with healthy controls and cocaine abusers by Sinha et al. 1992, 2002, 2005.

fMRI acquisition procedure.

All fMRI scans took place at the Yale Magnetic Resonance Research Center (MRRC) in New Haven, Connecticut. All images were acquired using a Siemens 3.0 T Trio system equipped with a standard quadature head coil, using T2-sensitive gradient-recalled single shot echo planar pulse sequence. Head positioning was standardized using the canthomeatal line and was secured with foam pillows and a band across the forehead. Subjects wore headphones and were fitted with a pulse-oximeter on their finger to obtain heart rate, recorded every 10 sec. Anatomical images of the functional slice locations were next obtained with spin echo imaging in the axial plane parallel to the AC-PC line with TR = 300 msec, TE = 2.5 msec, bandwidth = 300 Hz/pixel, flip angle = 60 degrees, field of view = 220x220 mm, matrix = 256x256, 32 slices with slice thickness = 4mm and no gap. Functional, blood oxygen level dependent (BOLD) signals were then acquired with a single-shot gradient echo planar imaging (EPI) sequence. Thirty-two axial slices parallel to the AC-PC line covering the whole brain were acquired with TR = 2,000 msec, TE = 25

msec, bandwidth = 2004 Hz/pixel, flip angle = 85 degrees, field of view = 220x220 mm, matrix = 64x64, 32 slices with slice thickness = 4mm and no gap, 190 measurements. At the end of the functional imaging, a high resolution 3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence (TR=2530 ms; echo time (TE) =3.34 ms; bandwidth=180 Hz/pixel; flip angle (FA) = 7°; slice thickness=1mm; field of view=256 x 256 mm; matrix=256 x 256) was used to acquire sagittal images for multi-subject registration.

fMRI imaging trials.

Four functional imaging trials (two stress and two neutral script runs) were acquired, lasting 5.5 min each. Each 5.5 min trial consisted of 1.5 min quite baseline (B) period, followed by a total 2.5 min guided imagery (I) period that included a 2 min read-image period and a 30 sec quite-image period, followed by a 1 min quite post-imagery period. Before and after the end of each scanning trial, subjects verbally rated their level of subjective distress to the question "how stressed or anxious are you feeling right now" on an auditory analog Likert scale ranging from 0-10. With the participants who were scanned in the between June and August of 2004, I guided the imagery trial and recorded all the reported data. I also entered all the recorded data in order to use it for the statistical analyses. After each trial, subjects also rated the imagery vividness on a 10-point scale. Between imaging trials subjects participated in progressive relaxation for 2 min to help reduce any leftover anxiety or distress from the previous trial. Subsequent trials were not initiated until the subjects' ratings of anxiety and pulse rate were stabilized down to their baseline levels. Using

this procedure, there were no differences in baseline and anxiety across trials.

Furthermore, all subjects had been trained in progressive relaxation and guided

imagery training procedure developed by Lang and colleagues [31]that has been used

in our previous studies [32, 33, 35] and described in detail previously by Sinha et al.

2003.

fMRI data analysis.

All data were converted from Digital Imaging and Communication in

Medicine (DICOM) format to analyze format using XMedCon. During the

conversion process, the first ten images at the beginning of each of the four functional

series were discarded to enable the signal to achieve steady-state equilibrium between

radio frequency pulsing and relaxation leaving 180 measurements for analysis.

Images were motion corrected for three translational and three rotational directions

[36]. Trials with linear motion in excess of 1.5 mm or rotation greater than 2 degrees

were discarded. No subjects were excluded secondary to the motion. Individual

subject data was analyzed using a General Linear Model (GLM) on each voxel in the

entire brain volume with a regressor specific for the task. The regressor was the

block of time while the subjects were listening to the particular script (as compared to

the baseline resting period). The resulting functional images for each script type were

spatially smoothed with an 8.08 mm Gaussian kernel to account for variations in the

location of activation across subjects. The output maps were normalized beta-maps,

which were in the acquired space (3.44mm x 3.44mm x 4mm).

To take these data into a common reference space, three registrations were calculated within the Yale BioImage Suite software package (http://www.bioimagesuite.org/, Duncan et al 2004). The first registration performs a linear registration between the individual subject raw functional image and that subject's 2D anatomical image. The 2D anatomical image is then linearly registered to the individual's 3D anatomical image. The 3D differs from the 2D in that it has a 1x1x1 mm resolution whereas the 2D z-dimension is set by slice-thickness and its x-y dimensions are set by voxel size. Finally, a non-linear registration is computed between the individual 3D anatomical image and a reference 3D image. The reference brain used was the Colin27 Brain [37] which is in Montreal Neurological Institute (MNI) space and is commonly applied in SPM and other software packages. All three registrations were applied sequentially to the individual normalized beta-maps to bring all data into the common reference space.

There are several possible ways that whole brain group comparisons can be conducted. First, each subject's stress difference map imagery – baseline (I-B stress) could be subtracted from the neutral difference map (I-B neutral), and the dual change maps can be contrasted to examine group differences, so called "double subtraction". In a different approach, each subject's difference map for each condition can be contrasted to examine group differences, i.e., both the stress and the neutral condition maps are contrasted separately across groups. We selected the former approach. We used the dual change maps comparing two groups at a time (e.g. follicular vs. luteal, follicular vs. men, luteal vs. men) in order to obtain a more complete information about emotional stress response differences across the menstrual cycle in women, and

in women vs. men. I participated in the processing of the images, establishing an suitable threshold level for the statistical analysis and ensuring that an appropriate combination of the imagery and subjects for the analysis.

According to Phillips et al 2003a, certain brain regions known to be involved in emotion processing can be defined as regions of interest (ROI) and were hypothesized to be involved in our study. Those were medial prefrontal cortex (MPFC), insula and anterior cingulate. A region-of-interest (ROI) approach using the normalized beta-weights (an estimate of percent signal change) as a measure of activation was selected to test our hypothesis of association between insular cortex, anterior cingulate and reported anxiety level and heart rate. The anterior, posterior and median insular cortices were defined in each hemisphere on the reference brain using anatomical landmarks and the Yale BioImage Suite software as was described by Papademtris et al., 2006. Normalized beta-weights for response in the right and left ROI of the anterior, posterior and median insular cortex was obtained for each trial and averaged for the stress, and neutral trials within each group. Spearman's rho correlations were conducted between medial orbitofrontal cortex, anterior cingulate and insula activity and reported anxiety levels in all women and in each group of women separately.

Physiologic data analysis.

Comparisons of the heart rate and anxiety ratings to determine the presence of the statistically significant variance were done for stress and neutral imagery trials

comparing them to baseline within group, as well as for the stress imagery trials

between groups using Student's t test. All those statistics were done by me.

Results.

Behavioral and Physiologic Data:

The results of subjects' self reported anxiety level and recorder heart rate

suggested that the stress imagery successfully elicited perceivable stress levels (table

2-4). In all three groups' average self-report of anxiety and measured heart rate

increased during stress imagery compared with non-stress trials. In comparison of the

self-reported and physiological stress response between two groups of women during

stress imagery, results were not statistically significant in the perceived anxiety, but

significant in measured heart rate (table 5). Comparison of physiologic response of

men to those of women in their distinct phases of the menstrual cycle revealed that

there were no significant differences in the anxiety ratings in men vs. follicular or

luteal groups (table 6).

	B HR mean(SD)	N HR mean(SD)	S HR mean(SD)	B Anx mean(SD)	N Anx mean(SD)	S Anx mean(SD)
Follicular	65 (7.93)	64.5 (7.41)	66.4 (7.45)	0.222 (0.428)	0.444 (1.25)	2.94 (2.82)
Luteal	71.5 (9.25)	70.4 (8.38)	74.6 (12.0)	0.950 (1.28)	0.800 (1.51)	2.95 (1.96)
Men	68.1 (7.62)	67.8 (8.05)	68.9 (8.70)	0.700(1.42)	0.500(1.00)	3.65 (2.30)

Table 2. Measured heart rate (HR) and self-reported anxiety ratings (Anx) during baseline (B), neutral (N) and stress (S) imagery in follicular and luteal group and in men.

Group	N-B HR			S-B HR			S-N HR		
	t	sdev	p	t	sdev	p	t	sdev	p
Follicular	-1.47	7.94	0.14	2.19	7.66	0.029	3.09	7.43	0.002
Luteal	-1.40	8.77	0.16	3.20	10.9	0.002	4.85	10.4	<0.001
Men	-0.438	7.86	0.66	1.21	8.23	0.23	1.97	8.47	0.05

Table 3. Comparison of heart rate by group in neutral vs. baseline trial (N-B), stress imagery vs. baseline (S-B), and stress vs. neutral imagery (S-N).

Group	N-B Anx			S-B Anx			S-N Anx		
	t	sdev	p	t	sdev	p	t	sdev	p
Follicular	0.705	0.932	0.48	4.05	2.02	0.003	3.44	2.18	0.002
Luteal	-0.340	1.40	0.74	3.82	1.65	0.001	3.89	1.75	<0.001
Men	-0.515	1.23	0.61	4.88	1.91	<0.001	5.62	1.72	<0.001

Table 4. Comparison of self-reported anxiety rating (0-10) by group in neutral vs. baseline trial (N-B), stress imagery vs. baseline (S-B), and stress vs. neutral imagery (S-N).

Luteal – Follicular Stress Imagery			
	t	sdev	p
HR	9.57	10.1	0.001
Anx	0.71E-02	2.40	0.99

Table 5. Comparison of self-reported anxiety rating (0-10) and recorded heart rate during stress imagery between luteal and follicular groups.

Men vs. Women Anxiety, Stress Trials			
	t	sdev	p
M-F	0.849	2.56	0.40
M-L	1.04	2.14	0.31

Table 6. Comparison of self-reported anxiety rating (0-10) and recorded heart rate during stress imagery between men and women in their follicular and luteal phases.

Imaging Data. Comparison between menstrual cycle phases.

Because the first goal of this study was to assess menstrual cycle influence on emotional stress response, the main comparison of interest was between that follicular and luteal phases. Nine women were scanned during their follicular phase and ten women were scanned in their luteal phase. As discussed above, participants did not vary significantly in their age, race, or education level. As predicted, we found that previously described stress circuitry, i.e. prefrontal cortex, insula, anterior cingulate and hippocampus, were modulated by menstrual cycle phase. Indeed, in follicular phase during stress imagery compared to neutral trials, right medial orbitofrontal cortex and bilateral anterior insula were activated more than during luteal, while luteal group had a greater level of activation in right anterior cingulate and left hippocampus. The difference held at p=0.01 (fig 1, table 7). No changes were observed in the lateral prefrontal cortex or amygdala.

Between-sex comparison. We compared our male subjects (n=10) to both groups of women separately. While comparing males to follicular group, males showed overall greater BOLD response, in particular in the areas of right medial prefrontal, hippocampus and dorsal anterior cingulate, and in the left inferior

temporal lobe (fig 2, table 8). Similarly, in the comparison of males to females in their luteal phase, males showed a greater degree of overall activation. Regions that had significant differences were in the right insular and prefrontal cortices and bilateral hippocampi (fig 3, table 9), with men showing greater BOLD response. We did not observe any regions in during stress imagery where women in either phased showed greater cerebral blood flow.

Correlation with heart rate and anxiety in women. To investigate a possible correlation of variations in physiologic data and activation of brain regions in women, we examined a relationship of level of activation of the insular, orbitofrontal and anterior cingulated cortices and hippocampus with recorded heart rate and anxiety reports of participants. Correlations were only done in the regions reported in our study as having differences in activation under emotional stress across menstrual. In the luteal group, during emotional stress, positive correlation was found between activation of the insular cortex and anxiety rating (fig 4). This correlation was also present when we used all female participants for the analysis (fig 5), however, that was likely secondary to the contribution by the luteal group, since no correlation of the self-reported anxiety and insular cortex activation was observed in the females in their follicular phase.
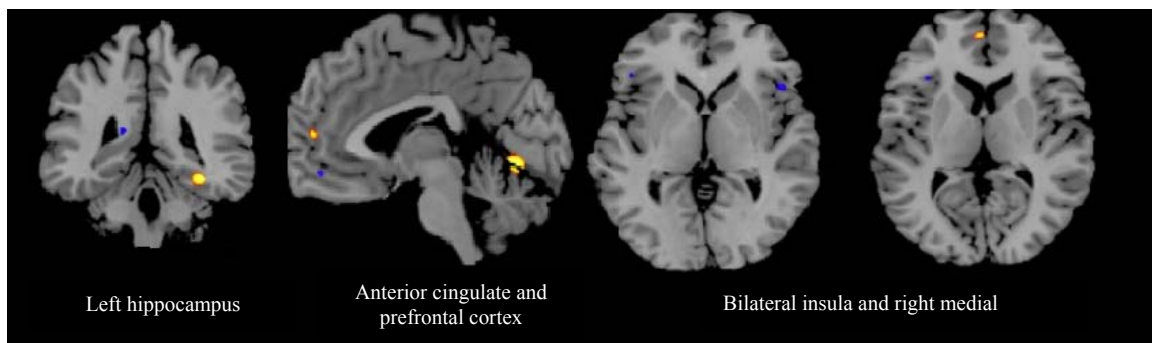
Fig. 1. Cross-menstrual cycle phase differences in BOLD response during stress compared to neutral imagery. Greater BOLD response during follicular phase in right medial prefrontal and bilateral anterior insula. Greater BOLD response during luteal phase in right anterior cingulate and left hippocampus. P=0.01
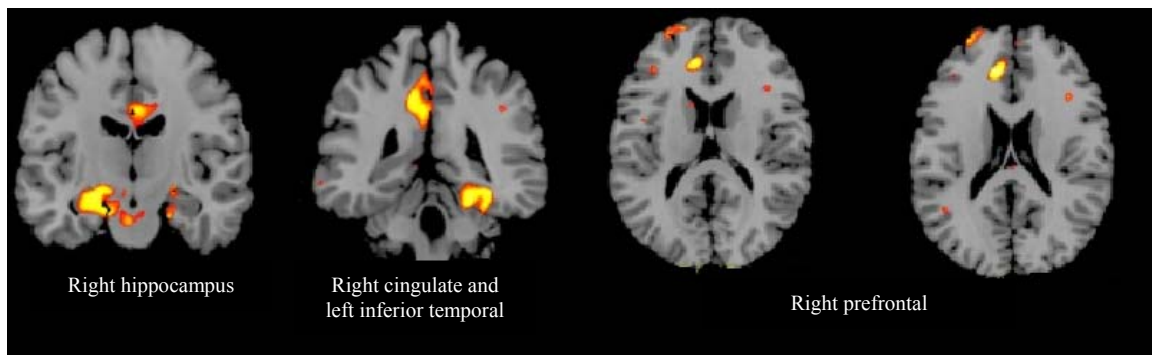

Fig. 2. Between-sex differences in BOLD response in males vs. females in the follicular phase during stress compared to neutral imagery. Greater BOLD response in males overall. Specific regions of increased blood flow in men are right hippocampus, right cingulate, right prefrontal cortex and left inferior temporal. P=0.01
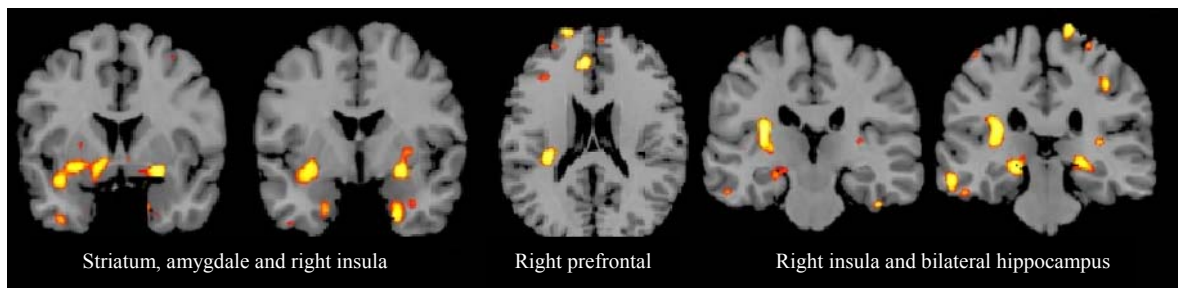

Fig. 3. Between-sex differences in BOLD response in males vs. females in the luteal phase during stress compared to neutral imagery. Greater BOLD response in males overall. Specific regions of increased blood flow in men are right prefrontal cortex, bilateral striatum, hippocampi and insular cortices. P=0.01

Region	Talairach Coordinate		
	x	y	z
ACC (BA 32)	3	49	9
OFC (BA 10)	5	44	-11
Hipp (BA 37, GF)	-33	-38	-11
Ins L (BA 45)	-47	17	4
Ins R (BA 47)	44	28	0

Table 7. Stress imagery vs. neutral. Cross-menstrual cycle analysis. P=0.01
Presented are the results of the contrast: activation during stress imagery vs. neutral imagery in
follicular phase vs. luteal with a p=0.01. Abbreviations for all tables: BA=Brodmann area,
ACC=anterior cingulate cortex, L=left, R=right, GF – fusiform gyrus, Hipp=hippocampus,
Amyg=amygdala, CG=cingulate gyrus, PCG=posterior cingulate gyrus, TMP=temporal lobe.

Region	Talairach Coordinate		
	x	y	z
Amyg	20	-5	-15
Hipp R	26	-16	-12
Hipp L	-23	-17	-13
CG R (BA 31)	4	-7	30
CG L (BA 31)	-6	-19	32
PCG R (BA 31)	6	-38	38

Table 8. Stress imagery vs. neutral between-sex differences. Presented are results of the contrast:
activation during stress imagery vs. neutral imagery in men vs. women in their follicular phase with
p=0.01.

Region	Talairach Coordinate		
	x	y	z
ACC (BA 31, 32)	-8	19	28
Ins R (BA 41)	32	-26	14
TMP (BA 21)	56	-30	-13
Putamen R	25	-2	-3
Hipp R	27	-17	-9
Putamen L	-24	-1	-4
Hipp L	-25	-16	-9

Table 9. Stress imagery vs. neutral between-sex differences. Presented are the results of the contrast:
activation during stress imagery vs. neutral imagery in men vs. women in their luteal phase.
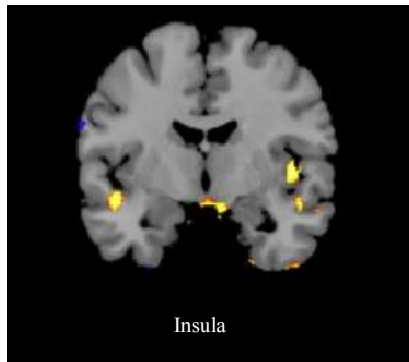
Fig. 4. Correlation between brain activity during stress imagery and subjective anxiety rating during the ***luteal*** phase of the menstrual cycle. During luteal phase stress imagery self-reported anxiety of the female subjects positively correlated with activity in the insular cortex. Coordinates (Ins L): x=-43 , y=-8 , z=4 . P=0.05
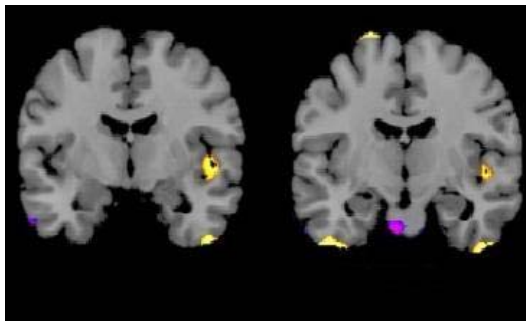

Fig. 5. Correlation between brain activity during stress imagery and subjective anxiety rating in *all women* regardless of the phase of the menstrual cycle. During stress imagery self-reported anxiety of the female subjects positively correlated with activity in the insular cortex. Coordinates (Ins L): x=-43 , y=-10 , z=4. P=0.05

Discussion.

Overall Activation.

The main results of this study support a menstrual cycle specific neural activation model underlying the central stress response elicited by stress imagery. They feature differences in activation of the cortical and corticolimbic structures in the follicular and in luteal phases. Our results also show differences in the stress response when contrasting male and female subjects at different points of their menstrual cycle (Fig. 1-3). The data shows more similar pattern in activation in men and women who are at the beginning of the menstrual cycle (follicular phase), than men and women in their luteal stage. Regression analysis approach was primarily employed to probe activation of the regions of interest involved in emotional and stress processing in women and correlate it to the self reported behavioral and measured physiological data. To our knowledge this is the first study to examine the effects of menstrual cycle on emotional stress responses when comparing to a non-stress control imagery control condition.

The following sections discuss the present finding in the context of the existing knowledge about hormonal and gender specific differences in stress.

Women Follicular vs. Luteal Phases.
Overall differences in cross-cycle brain activation.

While comparing central neural activation in women in their follicular and luteal phases in response to emotional stress compared to the neutral imagery, our

results indicate differences in activation of the cortical and corticolimbic structures (Fig. 1). When exposed to the stress imagery, women in the follicular phase showed greater activity in the insular and medial prefrontal cortex, while women in the luteal phase showed greater BOLD response in the anterior cingulate and hippocampus.

There are previous reports of differential activation of the central neural structures depending on the hormonal status of the subjects. However, these studies have mostly focused on cognitive tasks [25, 26, 38, 39] and reward processing [40], and attributed differences in activation to changing levels of estrogen, progesterone, or both. While the data revealing the influence of the ovarian hormones on neural activation is present, the reports on specific actions of estrogen and progesterone are controversial. Some researchers report increased level of activation in the presence of estrogen especially in the areas of midbrain, striatum and frontal cortex [25, 40], and others report attenuation of arousal particularly in the amygdala, brainstem, orbitofrontal cortex and anterior cingulate [41]. Cerebral asymmetries during various tasks are also seem to be affected by the circulating levels of hormonal steroids, implicating progesterone [42] or estrogen and progesterone [39] as having the main influence. In our results we did not observe a dramatic lateralization response to the emotional stress response across the menstrual cycle. We observed bilateral differences in the BOLD response in insula, however, the changes in the activation of medial prefrontal and anterior cingulate cortices were confined to the right hemisphere, and changes in hippocampus occurred on the left.

All the regions that showed variability in the activation during stress imagery, have been previously reported to be involved in processing of stress responses [12,

13, 19], however, our results are the first to show variation in the activation of those regions under emotional stress at different points of the menstrual cycle. It is possible that either estrogen or progesterone may be contributing to the differences in brain activations. While we used the level of estrogen and progesterone of our subjects with a goal to confirm the day of the menstrual cycle during imaging procedure, those measurements were not taken on the day of the fMRI and therefore we were not able to investigate a possible correlation between hormonal levels and central neural activation pattern. In the past, it has been argued, however, that it is estrogen or ratio of estrogen/androgens that influences arousal in women [41]. Although other pituitary and gonadotropins also show cyclical changes in women, it has been suggested that ovarian steroids are the ones to exhibit greater influence on the brain activity [41, 43, 44].

The interactions of gonadal steroids and neuroendocrine stress system have been shown in the past. With stress exposure women in their luteal phase tend to exhibit similar level of salivary cortisol response to men [22, 23], with women in the beginning of the cycle showing responses of a smaller magnitude [21]. More specifically interactions of estrogen and HPA axis have been reported, with estrogen influencing the transcription of the CRH promoter [45] and variations in intrinsic progesterone levels correlating with cyclical changes in GABAa receptor, and progesterone upregulating its expression [46]. The detection of estrogen receptors alpha and beta in hypothalamic nuclei, hippocampus, amygdala, and frontal cortex [47-51] provides the basis for the idea of altered expression of estrogen during arousal at specific phases of cycle as shown in the current study, and differences in

the levels of expression ER alpha mRNA in patients with affective disorders [50, 52]

raises the possibility estrogen's role in mood and mood disorders.

Cross-cycle differences in activation of the specific brain regions: insula, anterior cingulate, medial prefrontal cortex and hippocampus.

All the regions in our study that showed cycle-related variation in activation under emotional stress were reported to participate in the emotional processing, comprising parts of so-called ventral or dorsal systems as proposed by Phillips et al. 2003a. After examination of animal and human lesion and neuroimaging studies they suggested that emotion processing is a complex process that involves several stages of identification of a stimulus, production of affective state in response to it, and regulation of the affective state. The network of corticolimbic structures involved in the first two processes – ventral system – includes amygdala, insula, ventral striatum, and ventral regions of the anterior cingulate and prefrontal cortices; the system predominantly regulation the affective state – dorsal – included hippocampus and dorsal regions of the anterior cingulate and prefrontal cortices. Given the results of our study and previously reported involvement of those structures in emotional stress response [12, 27] it is appropriate to briefly address the potential significance of each of those regions separately.

Medial prefrontal cortex provides frontal influence over autonomic and endocrine function [53] and integrates the function of bodily states and goal-directed behavior. It has been suggested in the past the medial OFC carries the representation

of emotionally valenced linguistic stimuli that changes across the menstrual cycle [27] and different levels of the BOLD response, greater in the follicular phase were found during arousal [41], results consistent with ours. In addition it has been hypothesized the ventral prefrontal regions modulate amygdalar and pathologic limbic activity in depression [18], disorder much more prevalent in women. Also, morphological variations of this region were related to certain forms of depression [54], which makes it possible to hypothesize that variation in response of that region during emotional stress depending on the hormonal status of females is related to their higher predisposition for depression.

Hippocampus. Reports that estrogen receptors have been located in the hippocampus [55, 56] are consistent with the repots that sex hormones such as estrogen can alter the excitability of hippocampal cells, as well as varied excitability of hippocampal cells depending on the phase of the menstrual cycle [57]. Ovarian hormones also strongly influence dendritic spine density in the CA1 region of the female hippocampus, effect specific to females, as estradiol-treated males fail to show increase hippocampal spine density [58]. In terms of emotional processing hippocampus have been implicated to be one of the regions to regulate the emotional state, where cognitive processes are integrated with and can be biased by emotional input, supporting effortful regulation of the affective states [15]. With our results demonstrating greater BOLD change in the hippocampus in the luteal phase it is possible that more effort is required to cope with the emotional stress premenstrually.

Anterior cingulate. In our study we observed increased cerebral blood flow in the anterior cingulate cortex in women during luteal phase during emotional stress. In the past this particular region has been linked multiple times to the emotional and stress processing in both healthy controls as well as patients with mood disorders. Supporting our results, reports of increased activity in the anterior cingulate in the premenstrual phase during inhibition task [59, 60] and during expectation of the unpleasant stimuli [61] have been made, implicating it in the task of emotional self-regulation. Originally, greater activity in the anterior cingulate during anticipation of the unpleasant and painful stimuli in women compared to men [62] and, also, increased activity within rostral anterior cingulate gyrus to negative emotional stimuli in patients with major depressive disorder has been demonstrated [18, 63]. More interestingly, one of the recent studies by van Reekum and colleagues in 2007 attempted to evaluate the relationship of anterior cingulate and psychological well-being. Their results showed that people with higher psychological well being had an increased activation in the ventral ACC for negative relative to neutral information as well as slower evaluation of the negative information [64], which showed that those subject could effectively recruit the ACC when confronted with potentially aversive stimuli, reducing activity in other subcortical regions and increase their psychological well-being by perceiving the negative stimuli as less salient (manifested by a reduced evaluation speed). Given our results describing a different activation of the anterior cingulate in women depending on the phase of the menstrual cycle it is possible that luteal phase is associated with better recruitment of the anterior cingulate and appraisal of the negative stimuli as less aversive and stressful. Those reports suggest

that anterior cingulate cortex is heavily associated with negative emotion processing and cognitive aspect of modulation of the affective state and possibly association of those mechanisms with menstrual cycle.

Insula. We observed that insula had an increased level of activation in the follicular stage during stress imagery, with levels of BOLD comparable to those of men; while it did not seem to be activated to the same extend by stressful stimuli in luteal phase. In the past insula have been shown to be activated during the anticipation of unpleasant stimuli [61, 65], disgust and mutilation pictures [66]. Certain studies have also highlighted the role of insula in recall of internally generated emotions [67]. Interestingly, increased activity in bilateral insular cortex has been observed in anxiety-prone individuals compared to normal controls in response to emotional stimuli [68]. Symptom provocation in individuals with obsessive-compulsive disorder, simple phobia, or posttraumatic stress disorder has been shown to be associated with increased cerebral blood flow in bilateral insular cortex [69, 70]. It has been hypothesized in the past that individual prone to anxiety have an altered interoceptive prediction signal, i.e. they experience an augmented difference between the observed and expected body state [71]. Given that in the current study women in the follicular and luteal phases did not report different levels of subjective anxiety during stress exposure, greater insula activity during the follicular phase could suggest greater sensitivity to interoceptive cues during this period in contrast to the luteal phase.

Anxiety and insula correlation.

While some studies tend to underemphasize the observation that bran regions implicated in emotional processing are involved in control of autonomic responses and peripheral arousal states as was pointed by Critchley et. al. 2003, a strength of this study is the availability of subjective and physiological responses concurrent with brain response data. Previous research that attempted to investigate this question, documents regions of medial prefrontal, anterior cingulate and insular cortices to be involved in detection and integration of peripheral autonomic responses and physiological condition of the entire body, thereby providing neural representation of interoceptive state [72].

We were most interested in the levels of perceived anxiety in women during stress imagery and concurrent brain activation. While we did not find a correlation between levels of reported anxiety and BOLD response in anterior cingulate or medal prefrontal cortices, we observed significant correlation of the posterior insula and anxiety in the luteal phase (Fig. 4), which was not present in the follicular phase. Interestingly, our female subjects did not report differences in the subjective anxiety ratings depending on the phase of the menstrual cycle. To discuss these finding it is necessary to first briefly review what is known about the basis of anxiety, interoception and corticolimbic structures that participate in the regulation of those processes. Interoception is the sense of the physiological condition of the entire body, and the neural systems involved in integrating such afferent signals to an internal representation of the current state. It involves the midbrain reticular nuclei, thalamus, and the posterior (interoceptive) cortex, which is integrated in the anterior insular

cortex [73]. The insular cortex has multiple bidirectional connections with the orbitofrontal cortex [74]. Insula, therefore, is centrally placed in order to process and integrate information about the environment and how it will affect the perceived body state [71]. From the anterior insula the information about the interoceptive state is relayed to the anterior cingulate, which is thought to evaluate the difference between a predicted and observed body state and event outcome, and to indicate the necessary level of the attentional resource in order to adjust behavior or cognition [75]. As it was hypothesized and discussed by Paulus and colleagues in 2006, it is the altered signal of an impending aversive body state that provides the basic link between altered interoception and anxiety, where the predicted and observed states of the body are vastly different, and anxiety state ultimately originates from experiencing an augmented signal difference between the observed and the expected body states.

In our study, even though the self-reported anxiety rating did not vary significantly across the menstrual cycle, the correlation between the anxiety rating and posterior insula activation was observed in the luteal (fig. 4), but not follicular phase. Also, the activation of the anterior insular cortex showed greater BOLD response during emotional stress in the follicular phase (fig. 1), with levels of the cerebral blood flow comparable to those of men. Given the results of the current study and the previously available information, it is possible to hypothesize that the corticolimbic structures that showed differences in activation during stress imagery across the menstrual cycle, same structures that were previously reported to be involved in the emotional and stress processing [12, 13, 19], are part of the neural circuits that also target processing and integration of the differences in the

interoceptive state to regulate anxiety increases across the menstrual cycle. It is possible that perception of the internal body state varies across menstrual cycle, requiring adjustments in the integration and regulation of this information. Given that only women in their luteal phase showed a relationship between posterior insula and anxiety and increase blood flow in the anterior cingulate, and women in the follicular phase had increase BOLD response in anterior insula, we can provide two different explanations for these findings. First, either women in luteal phase had a higher awareness on the internal state of their bodies, reflecting it in the activation of the posterior insula, and requiring a greater engagement of the region that deploys attentional resources in order to modulate cognitive response and behavior, i.e. anterior cingulate. Second possibility, is that anterior insular cortex is simply more engaged in regulation of the interoceptive state and anxiety in the follicular phase, down-regulating activity of the posterior insula, which lowers the necessity to recruit a higher executive structure, i.e. anterior cingulate, in order to process the difference in the expected and the actual body state and regulate subjective anxiety.

It is obvious that in order to support or disprove either of these hypothesis further studies will be necessary to further investigate the activation of the involved structures and their correlation with anxiety. However, previous research documents a relationship between insular cortex and anxiety. Altered insular function has already been described in patients suffering from various anxiety disorders. It has been shown that anxiety-prone individuals have increased insula activation during emotional processing [68], while patients with social phobia show insular blood flow decrease during a public speaking task [76]. Trials of such pharmaceutical agents as citaprolam

and lorazepam in patients with anxiety disorders also seem to affect the activation of the insular cortex while reducing the symptoms [77, 78], and according to Wise and colleagues, 2007, midasolam reduced activity in the anterior insula preceding painful stimuli [79]. Interestingly women have higher prevalence of anxiety disorders than men. While our results do not explain this phenomenon, they could potentially serve as the first step to understanding its neural basis and future directions for treatment.

Men vs. Women.

Overall differences in between-sex brain activation.

While fight-or flight response is generally regarded as the prototypic human response to stress, characterized by sympathetic nervous system activation and hormonal cascade that results in the secretion of catecholamines, increased focus and fear [80, 81], and supported by studies in predominantly male subjects, it seems that from the evolutionary perspective alternative behavioral responses would likely have evolved in females. The female of the species makes a greater investment in pregnancy and nursing, and that should lead to selection for female stress responses that do not jeopardize the health of the mother and the offspring, and that maximize the likelihood that they will survive [82]. That would also mean a possibly blunted HPA response, focusing stress response on attachment and caregiving processes influenced by oxytocin, variation previously observed by several investigators [7]. Although we did not measure the cortisol response at the time of stress imagery, we observed a greater degree of central neural activation in males, with no regions of

increased BOLD response in females under emotional stress when compared to males. While the opposite results have been reported in the past, with females showing a greater degree of activation in response to the negative stimuli [83], our neuroimaging findings agree with the general trend of greater acute activation of HPA in males compared to females [7].

In particular male subjects had a more significant level of activation in prefrontal cortex, posterior cingulate, hippocampus and amygdala compared to females (Fig. 2,3). These finding are consistent with the idea of "fight-or flight" response to stressors in males. Greater activation of the right prefrontal cortex – area which is important in vigilance – during stress response in males has been reported previously [19], and the association between negative emotions and right prefrontal activation was originally based on electrophysiological findings [84-86], and further confirmed by fMRI data [13, 19]. Reports of the differences in cognitive performance while experiencing negative emotions between two genders have been made, reporting the activation of the parietal and prefrontal cortices in males – regions important for cognition and cognitive control [5]. In addition high levels of right-sided prefrontal activation have been linked with negative affective style and suppressed immune function [87, 88], which could be a plausible neural mechanism underlying negative health consequences including hypertension, substance abuse and immune suppression seen more often in men [10].

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In addition to the variation in cerebral blood flow of the prefrontal cortex, we also observed an increased activation of the hippocampus in males compared to

females in both phases of the menstrual cycle. While relatively unknown are hippocampal sex differences in the reaction to stress, it has been shown that in both rats and monkeys, chronic stress causes damage to the hippocampus in males, but does so far less, if at all in females [80]. It is possible, given our results, that male hippocampus either plays a greater role in the stress processing in males, or more susceptible to stress induced damage through a greater recruitment of that area. Chronic stress damage is widely known among neuroscientists in males, but far less so among females. It has been suggested that susceptibility of hippocampal cells to chronic stress has a role in two psychiatric conditions – post traumatic stress disorder (PTSD) and clinical depression [80], both of which have different gender-related prevalence. It is obvious that possible resistance of female hippocampal cells to stress-induced damage could play an interesting role in pathology and treatment of certain psychiatric conditions and demands consideration by anyone attempting to link stress-induced cell death to affective disease states.

Our group of males showed an increase in the amygdala activation when compared to females during stress imagery. Several studies now report sex influences on amygdala function, providing compelling evidence that the amygdala critically involved in enabling us to acquire and retain lasting memories of emotional experiences; the degree of activation of amygdala by emotional arousal during encoding of emotionally arousing material (both pleasant and unpleasant) and correlates highly with subsequent recall [89]. The involvement of amygdala in fear processing has been shown with greater and more consistent activation during

aversive stimuli than to positively valenced ones. It has been also hypothesized that activation of the amygdala is associated with modulation of motor readiness, autonomic function, and cognitive processes including attention and memory [90] – all the necessary components of the effective fight-or flight response to stress in males.

Differences in BOLD response in men vs. women at different points of the menstrual cycle.

In the past there has been very few imaging studies comparing brain activity in male to females during two distinct phases of the menstrual cycle. Studies that are available, chose to focus on motor or cognitive tasks [25, 26] such as word-stem-completion and mental rotation, reporting increased similarities in the activation pattern in males and women in the follicular phase, than in males and luteal. To our knowledge we are the first to investigate and report the differences in the central neural activation between males and females in two different phases of the hormonal cycle during emotional stress. Most brain regions revealed similar differences, however insula cortex showed variations, with women in follicular phase activating it to the same degree as males (Fig. 2), and greater than women in the luteal phase (Fig.1). This could be due to different neuronal or endothelial receptor concentrations, differences in synaptic function, or changes in the cerebrovascular anatomy in that region, but most likely to a combination of the above factors. However, given the association of insula and anxiety and differences in the interoceptive state of the subject [71], it is interesting that no significant statistical difference in self-reported

anxiety was observed between men and women in either phase. This could potentially support the idea of differences in correlation of the self-reported anxiety and insular cortex, depending on the hormonal status of females, with similar regulation of the insular activity in men and follicular phase. Undoubtedly, further investigation of the stress response and anxiety and their correlations with levels of the ovarian hormones would benefit a better understanding of the difference in stress regulation between sexes and provide a new gender-based approach for the treatment of anxiety-related psychiatric conditions.

Conclusion.

The present study revealed cycle-specific differences in healthy menstruating women in the neural response to emotional stress elicited by stress imagery, as well as the correlation of levels of self-reported anxiety and activation of the corticolimbic structures. The differences in cycle-dependent activation were consistent with the differences in the between-sex activation, when taking into account the phase of the menstrual cycle. The males had a greater overall activation consistent with the previous reports of the HPA axis levels of stress response. The cycle-dependent stress differences and the anxiety and insular activation in women in their luteal phase are consistent with the hypothesis of the insular involvement in the interoception and anxiety processing. Our results further adhere with the idea of the corticolimbic network that is involved in the stress response and anxiety regulation, where higher cortical structures integrate the information about current and expected body state to produce and manage an appropriate response. Given that current study is the first one to show the cycle-dependent correlation of anxiety and insular cortex activation, it could represent an initial step in the understanding the reasons for unequal stress response and anxiety-related psychiatric conditions in women and men.

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