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Sex- and sex hormone- related variations in energy-metabolic frontal brain asymmetries: A magnetic resonance spectroscopy study

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Abstract

Creatine is a key regulator of brain energy homeostasis, and well-balanced creatine metabolism is central in healthy brain functioning. Still, the variability of brain creatine metabolism is largely unattended in magnetic resonance spectroscopy (MRS) research. In the human brain, marginal sex differences in creatine levels have been found in the prefrontal cortex. It is however not known to what degree these sex differences are stable or change with varying gonadal hormone levels. The current study therefore investigated creatine in the prefrontal cortex across the menstrual cycle. In addition, we explored cerebral asymmetries. Creatine, Choline (Cho), N-acetylaspartate (NAA), Myo inositol (mI), and glutamate+glutamine (Glx) were assessed three times in 15 women and 14 men using MRS. Women were tested in cycle phases of varying hormone levels (menstrual, follicular, and luteal phase). Prefrontal creatine was found to change across the menstrual cycle, in a hemisphere-specific manner. Women in the follicular phase showed increased left prefrontal creatine accompanied with reduced right prefrontal creatine, while this asymmetry was not present in the luteal phase. In men, the creatine levels remained stable across three testing sessions. In general, both men and women were found to have higher creatine levels in the left as compared to the right prefrontal cortex. Exploratory analyses of other metabolites showed similar asymmetries in NAA, Cho, and mI, while Cho also showed a menstrual cycle effect. This is the first time that sex hormone-related changes in creatine metabolism have been demonstrated in the human brain. These findings may have important methodological implications for MRS research, as it supports previous concerns against uncritical usage of creatine as a reference measure for other metabolites, assumed to be invariant across individuals and conditions.

Key words: Creatine, MR spectroscopy, sex hormones, menstrual cycle, hemispheric asymmetry, prefrontal lobe

Introduction

Whereas the brain makes up 2% of the total body weight, it consumes about 20% of the produced energy (Attwell and Laughlin, 2001). Creatine acts as a buffer for adenosine triphosphate (ATP) and is a particularly convenient form of energy storage in tissues with high energetic needs, such as brain and muscle tissue (for reviews see Joncquel-Chevalier Curt et al., 2015; Wallimann et al., 1992). During low demand periods, ATP can be used to convert creatine to phosphocreatine as storage of energy. In turn energy can rapidly (anaerobicly) be released by the transfer of the phosphate group to adenosine diphosphate (ADP) making ATP (Owen and Sunram-Lea, 2011; Wallimann et al., 1992). Creatine is also an antioxidant (e.g. Joncquel-Chevalier Curt et al., 2015) and a neuromodulator (Chebib et al., 2009; Neu et al., 2002; Oliveira et al., 2008), although our understanding of the latter is currently limited (Almeida et al., 2006). Both diet and endogenous synthesis contribute to brain creatine levels. The liver and kidneys are important for creatine synthesis, and circulating creatine can to some extent pass the blood-brain barrier (Lyoo et al., 2003). However, the brain is also dependent on local synthesis (Braissant et al., 2001), and deficiencies in creatine metabolism are associated with cognitive impairment (Allen, 2012).

In the human brain the creatine levels can be assessed by proton magnetic resonance spectroscopy (^{1}H MRS). MRS is a non-invasive technique for obtaining in-vivo spectral information about the concentration of metabolites in the brain by taking advantage of the magnetic properties of the hydrogen nucleus (de Graaf, 2007). This technique is frequently used in basic and applied research as well as a diagnostic tool (Brandao and Domingues, 2004). Creatine is often used as a reference compound in MRS studies, incorporated as the denominator in metabolic ratios (e.g. Batra et al., 2008; Brandao and Domingues, 2004). However, the stability of creatine, and therefore the validity of this practice is questionable

(e.g. Dechent et al., 1999; Hamakawa et al., 1999; Rae, 2014). The conditions under which creatine may vary therefore needs further clarification.

In the human brain, there is evidence of sex differences in creatine levels in the prefrontal lobe (PFL), although this is so far inconclusive. While Chang et al. (2009) found a trend towards bilaterally higher creatine levels in frontal regions in women, Hamakawa et al. (1999) found marginally increased prefrontal levels only in the right hemisphere in males. However, neither of the studies controlled for the menstrual cycle, and it is therefore unknown whether these differences are related to stable sex-trait characteristics emerging from early life sex hormone exposure (organizing effects), or if they are state-dependent and variable with circulating gonadal hormone levels (activating effects; (McCarthy et al., 2012)). In non-brain tissues creatine kinase activity has been found to change across the rat estrous (Somjen et al., 1991) and human menstrual cycle (Thompson et al., 2006), and shown to be upregulated by estradiol (Somjen et al., 1989; Somjen et al., 1991), and in females using contraceptive pills (Paterson and Lawrence, 1972). It has also been found that neuroprotective effects of creatine are dependent on sex hormones, such that creatine and sex hormones act synergistically (Allen et al., 2015). More specifically, Allen et al. (2015) found that high creatine levels reduced depressive symptoms in gonadectomized female and male rats only when sex hormones were administered simultaneously.

Another branch of sex hormone research has shown that gonadal hormones (estradiol, progesterone, and testosterone) are potent modulators of hemispheric asymmetries (Alexander et al., 2002; Hodgetts et al., 2015; Joseph et al., 2012). Several cognitive functions, such as language and visuospatial processing (Beaton, 1985; Hugdahl and Davidson, 2003), and emotions (Ahern and Schwartz, 1979) are asymmetrically organized in the brain. For language, women typically reveal more leftward asymmetry during the follicular phase characterized by high estradiol levels (Altemus et al., 1989; Cowell et al., 2011), as compared

to the menstrual phase (Cowell et al., 2011) when hormone levels are generally low, and luteal phase (Alexander et al., 2002; Altemus et al., 1989), in which levels of both estradiol and progesterone are high (but see Hodgetts et al., 2015). Testosterone has especially been associated with organizing effects on the brain (Geschwind and Galaburda, 1985). Nevertheless, some studies suggest circulating testosterone to have activating effects across the diurnal (Sanders et al., 2002) and menstrual cycles (Schoning et al., 2007). Although the sex and sex-hormonal variations in functional brain asymmetries are well studied, there has been limited research into the metabolic, and particularly bioenergetic, basis of these asymmetries. Regarding creatine, one study suggests creatine to be asymmetrically distributed across the hemispheres in both females and males (Maudsley et al., 2009; but see Nagae-Poetscher et al., 2004; Pouwels and Frahm, 1998). Whether the leftward asymmetry in creatine previously observed (Hunter et al., 2005; Maudsley et al., 2009) varies with fluctuating levels of sex hormones across the menstrual cycle is currently unknown.

The present study aimed to investigate the effects of sex and sex hormones on creatine levels in the PFL and therefore tested women in three cycle phases (menstrual, follicular, and luteal) and men across three sessions. We hypothesized that women would show higher levels of creatine in the PFL as compared to men (Chang et al., 2009) and particularly in the cycle phases where hormone levels are high (follicular and luteal). Estradiol levels in particular were expected to correlate with creatine levels, but other gonadal hormones (testosterone and progesterone) were also explored. Furthermore, we aimed to explore asymmetry effects of creatine. Based on the findings of Maudsley et al. (2009), we expected prefrontal creatine to be higher in the left relative to the right hemisphere in both men and women. Asymmetries in creatine levels might also be expected to fluctuate across the menstrual cycle in a similar manner to that which has been shown for functional asymmetries (Altemus et al., 1989; Cowell et al., 2011).

Methods

Participants

Twenty-one women and fifteen men underwent three sessions of MRS acquisition during rest. Five women were excluded based on hormone measurements (see *Hormone assays*). In addition, one woman and one man were excluded due to poor quality spectra (see data analysis section).

In total, fifteen women (mean age $M = 23.25$, $SD = 5.01$ years) and fourteen men ($M = 23.13$, SD = 2.42 years) were included in the data analyses. All participants were native Norwegian speakers, and right handed (Edinburgh Handedness Inventory (Oldfield, 1971)). Mean laterality quotient was 92.89 ($SD = 11.4$) in women and 93.33 ($SD = 10.46$) in men. Women were tested once in each of three different cycle phases, that is, the menstrual phase (cycle day 2-4, low estradiol and progesterone), the follicular phase (cycle day 8-12, high estradiol, low progesterone), and the luteal phase (cycle day 20-22, high estradiol and progesterone). They had regular menstrual cycles with a mean cycle length of 26-32 days. None of the women had used hormone-regulating medications, including hormonal contraceptives, or had been pregnant, in the six months prior to the study. Of the fifteen women included, five started testing in their menstrual phase, five in their follicular phase, and five in their luteal phase. To control for potential influences of the circadian rhythm (Ahn et al., 2011), time of testing deviated no more than three hours between testing sessions. Men were tested three times with one to two weeks in between each testing sessions. For the analysis, they were randomly assigned into groups corresponding to the three female cycle phase groups. The study was approved by the Regional Committee for Medical Research

Ethics at the University of Bergen, and participants gave their informed consent according to the Declaration of Helsinki.

Hormone assays

Two saliva samples, one at the beginning and one at the end of each testing session were collected for all participants. The samples were analyzed for concentrations of estradiol, progesterone, and testosterone with luminescence assays by an independent hormone laboratory (IBL International). The two samples for each session were blended before analysis.

Luteal progesterone levels served as an indicator of ovulation in women. Fifteen women, whose progesterone and estradiol levels were within expected ranges of the respective cycle phases, were included for further analysis (see Table 1).

Table 1: Means, standard deviation, and range (in brackets) for estradiol, progesterone, and testosterone levels from saliva sample in the women (n=15) during the menstrual, follicular, and luteal cycle phase.

Hormone in pg/ml	Menstrual Phase	Follicular Phase	Luteal Phase
Progesterone	54.0 ±20.2 (25.2-91.5)	58.1 ±31.3 (23.6-136.0)	188.9 ±96.5 (95.2-416.7)
Estradiol	2.8 ±1.3 (1.3-5.3)	3.6 ±1.5 (1.6-6.3)	4.5 ±1.7 (2.1-7.7)
Testosterone	17.2 ± 5.8 (9.2-30.3)	17.2 ± 6.8 (7.3-30.1)	18.24 ± 7.5 (6.6-34.0)

A repeated measures ANOVA with progesterone levels as dependent variable revealed a significant effect of cycle phase ($F(2,28)= 32.21$, $p<0.001$, $\eta^2=0.70$). Tukeys HSD post-hoc test showed luteal progesterone levels to significantly differ from the menstrual ($p<0.001$) and follicular phase ($p<0.001$). The same ANOVA for estradiol levels also revealed a cycle phase effect ($F(2,28)=5.48$, $p=0.01$, $\eta^2=0.28$) with a significant difference between the menstrual

and luteal phase (p=0.01, Tukeys HSD). Repeated measures ANOVA on testosterone levels revealed no effect of cycle phase ($F(2,28)=0.16$, $p=0.85$, $\eta^2=0.01$).

MR acquisition

Data were collected with a 3 T GE-Signa MRI scanner. First, an anatomical T1-weighted image was acquired of each subject (3D fast spoiled gradient echo (FSPGR), repetition time (TR)/echo time (TE)/flip angle (FA)/field of view (FOV) 7.9 ms/3.2 ms/11°/256 mm, 256 × 256 scan matrix, 180 sagittal slices, voxel size 1 × 1 × 1 mm). Thereafter, MRS acquisition was performed. Short echo time ^{1}H-spectra were obtained from the left and the right PFL (see Figure 1) by using a single-voxel point-resolved spectroscopy (PRESS) sequence (voxel size 20 × 20 × 20 mm^3, TE/TR = 35 ms/1500, 128 repetitions) for the MRS measurements. As is standard on the GE implementation of the PRESS sequence, unsuppressed water reference spectra (eight repetitions) were acquired automatically after the acquisition of water-suppressed metabolite spectra, and averaged before being processed and analyzed. Voxel localization was performed using the T1 weighted structural image obtained prior to the MRS sequence. Repositioning of the voxel was performed in each session by placing the voxel with reference to anatomical landmarks in the frontal lobe. The voxels were placed in the left and right PFL since the prefrontal region has a high density of receptors for gonadal hormones (Gillies and McArthur, 2010; Montague et al., 2008). The voxels covered parts of inferior and middle frontal gyrus with associated white matter. Particularly the inferior frontal gyrus has shown functional lateralization in language (Specht, 2014). The proportion of gray and white matter covered by the voxel was on average 26,8% and 71,98%, respectively.

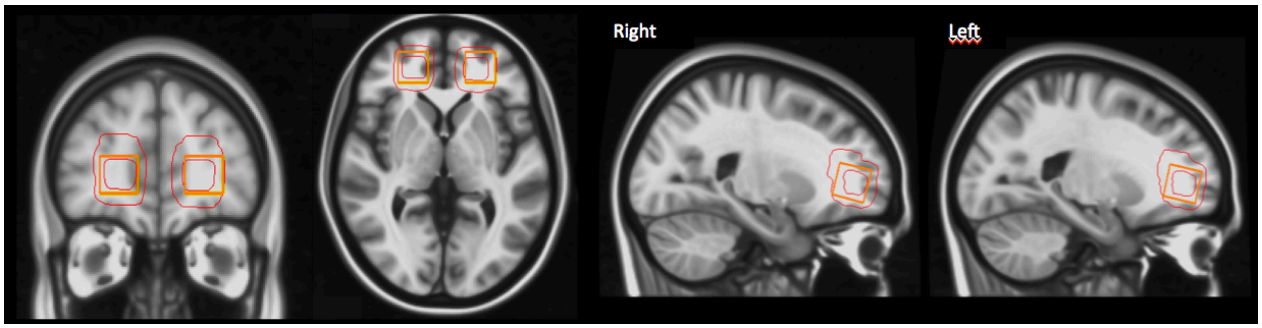

Figure 1: Placement of voxels in left and right prefrontal cortex depicted in coronal, horizontal and lateral views. The orange box represents the placement for one representative subject. The red contours indicate 60% and 95% confidence regions for placement across the entire group (when mapped to a standard template).

Data analysis

MRS data were analyzed using LCModel version 6.3-1J (Provencher, 1993) with a standard basis set incorporating components from 15 metabolites (Alanine, Aspartate, Creatine, γ-aminobutyric acid, Glucose, Glutamine, Glutamate, Glycerophosphorylcholine, Phosphorylcholine, Lactate, myo-inositol, N-acetylaspartate, N-acetylaspartylglutamate, scyllo-inositol and Taurine. Metabolite estimates were scaled to an internal water reference (obtained immediately following acquisition of metabolite data in each region), accounting for differing water concentration in the different tissue classes, partial volume effects, and metabolite relaxation times and differing water relaxation times between the tissue classes according to the equation, derived from Gasparovic (2006):

$$\frac{1}{\mathrm{frac}_{\mathrm{CSF}}}\left(\sum_{\mathrm{TC}=[\mathrm{GM},\mathrm{WM},\mathrm{CSF}]}\mathrm{frac}_{\mathrm{TC}}\cdot\mathrm{conc}_{\mathrm{water,TC}}\cdot\left(\frac{\left(1-e^{-\mathrm{TR}/\mathrm{T1}_{\mathrm{water,TC}}}\right)\cdot e^{-\mathrm{TE}/\mathrm{T2}_{\mathrm{water,TC}}}}{\left(1-e^{-\mathrm{TR}/\mathrm{T1}_{\mathrm{metab}}}\right)\cdot e^{-\mathrm{TE}/\mathrm{T2}_{\mathrm{metab}}}}\right)\right)$$

Parameters were $\mathrm{conc}_{\mathrm{water,GM,WM,CSF}}$=[43300,36080,54840] (Ernst et al., 1993; Gasparovic et al., 2006), $\mathrm{T1/T2}_{\mathrm{water\ GM,WM,CSF}}$=[1304/93,832/79.2, 3817/503] ms (Lu et al., 2005; Piechnik et al., 2009; Vymazal et al., 1999; Wansapura et al., 1999) $\mathrm{T1/T2}_{\mathrm{Ch}}$=1090/350ms, $\mathrm{T1/T2}_{\mathrm{Cr}}$=1460/152ms, $\mathrm{T1/T2}_{\mathrm{Glu}}$=1270/180ms, $\mathrm{T1/T2}_{\mathrm{mI}}$=1230/192ms,

$T1/T2_{NAA}$=1470/247ms (Ernst et al., 1993; Ganji et al., 2012; Gasparovic et al., 2006; Mlynarik et al., 2001; Rutgers and van der Grond, 2002)

Tissue content within the spectroscopy voxel was estimated from the T1 image using the segmentation functionality of the Statistical Parametric Mapping (SPM8) software (www.fil.ion.ucl.ac.uk/spm), with a voxel mask derived per subject from actual geometry using an in-house script. Results are presented as achieved ratios, and were not projected to 'pure' GM or WM values.

Quality control of the spectra was conducted and resulted in the identification of two poor spectra, belonging to two different subjects; all spectra from these subjects were excluded from statistical analysis. Previous studies (e.g. Slotboom et al., 2009) have raised concerns regarding the utility of basic fit metrics (CRLB %SD of estimates, linewidth, etc) as the sole basis for quality control, with hard limits on (for example) %SD of estimates introducing a possible source of bias. Therefore, we considered multiple factors in the assessment of spectra quality. Spectra were assigned a quality score, which weights multiple fundamental, scalar metrics (spectral linewidth (FWHM), signal-to-noise ratio (SNR) and CRLB %SD of estimates for key metabolites, summarized in supplementary tables S1 and S2) in addition to assessment of variance and magnitude of features in the residuals after fitting, and magnitude of aberrant features in the spectrum (relative to group mean spectrum). This quality score flagged spectra of concern, supplementing visual inspection of the fit and residuals, to identify spectra which were of insufficient quality for meaningful assessment. Typical spectral quality for a representative female and male subject is shown in Figure S1.Because of difficulties in differentiating creatine and phosphocreatine at 3-T magnetic field strength (de Graaf, 2007), the combination of the two is reported, and hereafter referred to as creatine. In other words, it is unknown what portion of the creatine pool that is in a phosphorylated form. The total creatine must therefore be interpreted as a potential for energy

storage. The MRS estimated creatine levels were subjected to a 2(Sex) by 3(Cycle Phase in women/Session in males) by 2(Hemisphere) repeated measures ANOVA. Post hoc analyses were performed using lower-level ANOVAs and thereafter Tukey HSD comparisons. Effect sizes are given as percentage explained variance (partial η^2). Correlation analyses were conducted to explore the relationship between creatine concentration and gonadal hormones. To reduce the number of variables, laterality indexes were calculated for creatine according to the formula [(L-R)/(L+R)]*100 in which L and R refer to hemisphere.

Exploratory analyses were conducted on other metabolites and included in supplementary material, namely N-acetylaspartate (NAA), Choline (Cho), Myo inositol (mI), and glutamate + glutamine (Glx). For obtained values and statistics refer to Table S1 (see attachement).

It has been suggested that creatine levels are unevenly distributed across gray and white matter (Brandao and Domingues, 2004). To make sure that the relative proportion of gray matter in the left and right voxels were not significantly different, a repeated measures ANOVA was conducted on gray matter values with the variables Sex, Cycle Phase/Session, and Hemisphere.

Results

Hemisphere and menstrual cycle effects in creatine

The 2(Sex) by 3(female Cycle Phase/male Session) by 2(Hemisphere) repeated measures ANOVA on creatine levels (see Figure 2) revealed a main effect of Hemisphere ($F(1,27)=16.02$, $p<0.001$, $\eta^2=0.37$) with higher creatine concentration in the left PFL (M = 5.48, SD = 0.66) than the right PFL (M = 4.87, SD = 0.66). Furthermore, a significant

interaction of Sex, Cycle Phase/Session, and Hemisphere was found ($F(2,54)=4.3$, $p=0.02$, $\eta^2=0.14$; see Figure 2). No other main or interaction effects were significant (all $F<1.1$, $p>0.36$, $\eta^2<0.04$). To explore the nature of the significant 3-way interaction, ANOVAs with the factors Cycle Phase/Session and Hemisphere were calculated separately for women and men. The main effect of Hemisphere was significant in both men ($F(1,13)=6.01$, $p=0.03$, $\eta^2=0.32$) and women ($F(1,14)=10.29$, $p=0.01$, $\eta^2=0.42$). While the ANOVA for men showed no further effects (all $F<0.78$, $p>0.48$, $\eta^2<0.05$), the ANOVA for the women revealed an interaction of Cycle Phase and Hemisphere ($F(2,28) = 5.13$, $p = 0.01$, $\eta^2=0.27$). To explore the 2-way interaction in women, post-hoc testing between all groups was conducted using Tukey's HSD. A significant difference between the left and right cerebral hemispheres was found in the follicular phase ($p<0.001$), and the menstrual phase ($p=0.04$), but not the luteal phase ($p=0.82$). No significant differences were found between female cycle phases within left hemisphere and right hemisphere, or between men and women.

Control analyses of gray matter

The 2(Sex) by 3(Cycle Phase/Session) by 2(Hemisphere) ANOVA on gray matter voxel content revealed no main or interaction effects (all $F<3.55$, $p>0.07$, $\eta^2<0.12$).

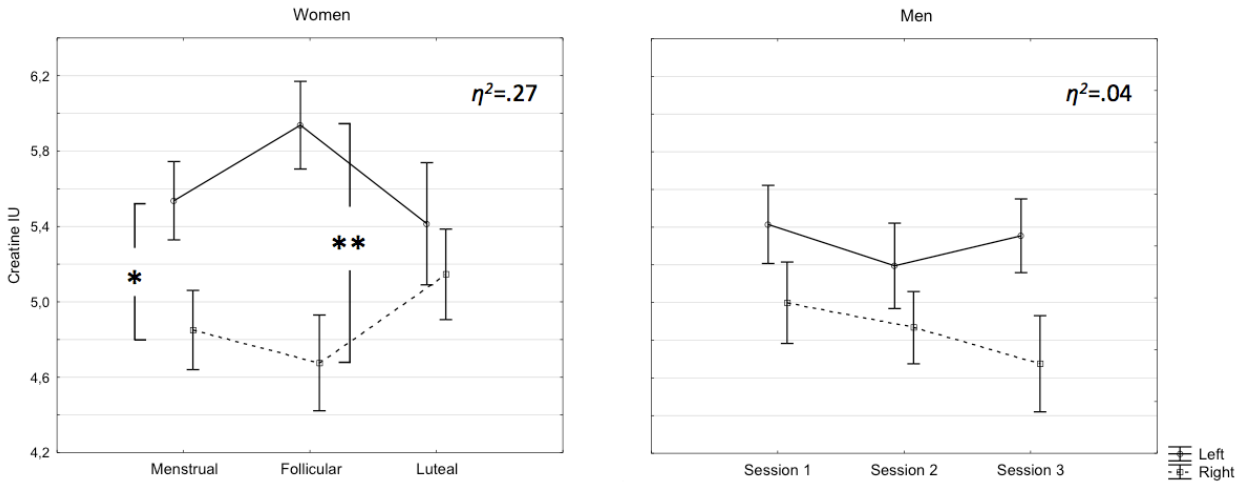

Figure 2: Creatine levels (y-axis) in left and right prefrontal cortex for women across the menstrual cycle phases (menstrual, follicular, and luteal), and across three testing sessions in men. Results are from the follow-up ANOVAs conducted for women and men with the variables Cycle Phase/Session and Hemisphere. Solid lines represent left hemisphere and dotted lines right hemisphere. Error bars indicate standard error. * indicates significant post-hoc comparisons at $p < 0.05$, and ** at $p < 0.001$, conducted to explore the significant interaction of Sex, Cycle Phase, and Hemisphere. For details please refer to text. Abbreviations: IU – institutional units.

Correlations between hormones and creatine

For descriptive purposes scatter plots of correlation between the different sex hormones measured (estradiol, testosterone, and progesterone) and creatine asymmetry were included (see Figure 3). A significant positive correlation was found between follicular testosterone and leftward asymmetry in creatine (p=0.03; r=.54), and luteal estradiol and leftward creatine asymmetry (p=0.05, r=.52) when not correcting for multiple comparisons (corrected alpha level = 0.006). No further significant correlations were found (All p>0.05; r<.51).

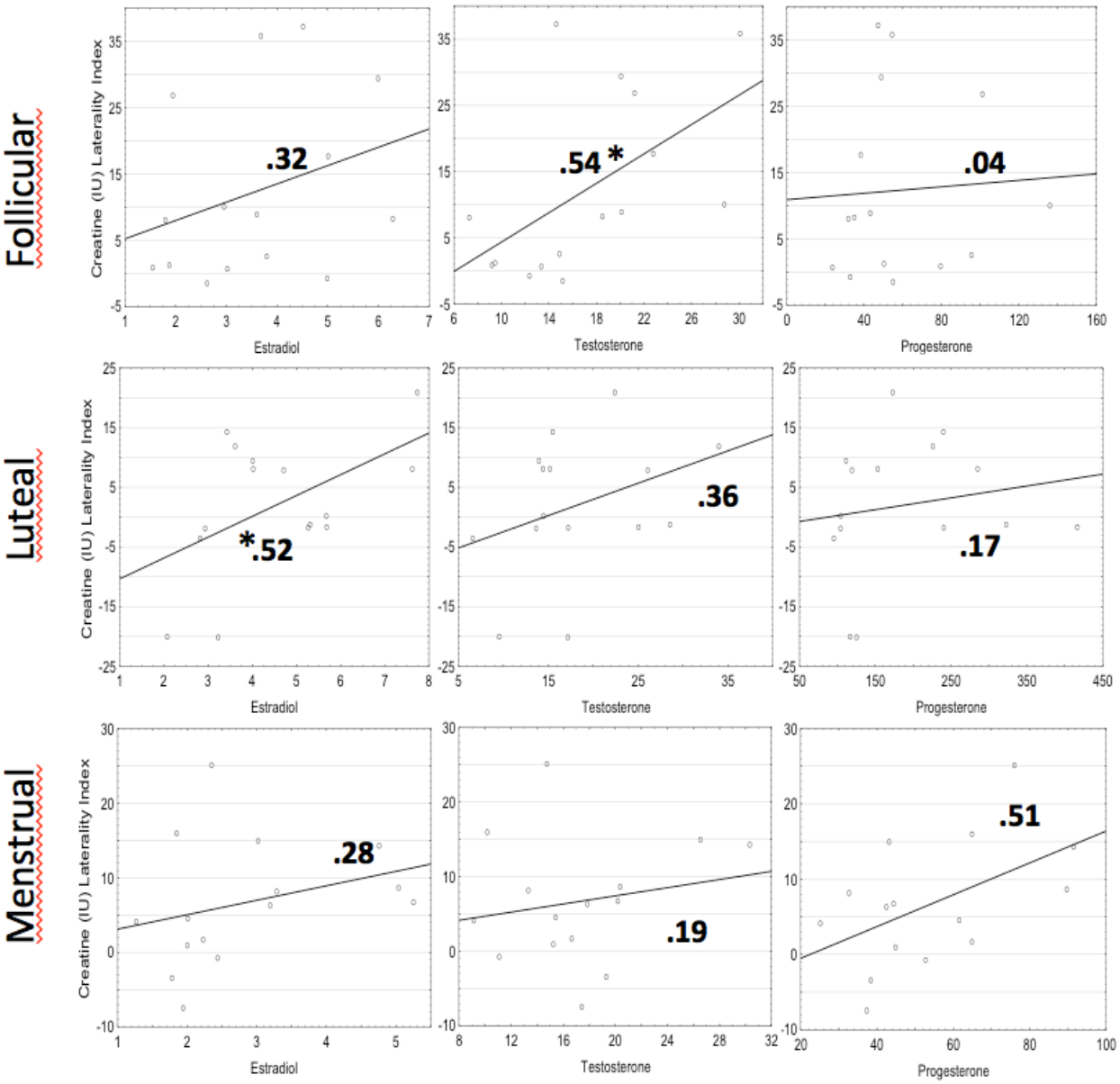

Figure 3: a) Descriptive statistics of relationships between the measured sex hormones (estradiol, testosterone, and progesterone on the x-axis (pg/ml)) and creatine asymmetry (y-axis) in the different cycle phases (follicular, luteal, and menstrual). Creatine laterality index represent degree of hemispheric asymmetry where positive values means bias to the left hemisphere and negative values means bias to the right hemisphere. Note that the values on y-axis and x-axis differ between cycle phases. Asterisk indicate significant correlations (*$p<0.05$). Neither of the correlations in figure 3 is significant when adjusting the alpha level for multiple comparisons. Abbreviations: IU = institutional units.

Discussion

The current study investigated prefrontal creatine levels in left and right cerebral hemispheres across the menstrual cycle, also comparing women and men (that were re-tested three times). The results showed overall (across sexes and cycle phases) higher levels of creatine in the left PFL as compared to the right PFL. In addition, the left prefrontal creatine levels were found to be especially high in women tested in the follicular phase, accompanied with a corresponding drop in the right prefrontal creatine levels. In women, this resulted in a significant asymmetry in creatine levels between left and right PFL in the follicular phase that was not present in the luteal phase. The creatine levels in men remained stable across testing sessions.

Creatine asymmetries

In line with our hypothesis, overall higher creatine levels were found in the left as compared to the right PFL. Control analysis of structural MR data suggested no significant difference in the amount of gray matter within the left and right MRS voxel, and it is therefore unlikely that the results are biased by differences in left and right voxel tissue composition. The results are in line with studies that reported higher creatine levels in the left hemisphere, including the frontal lobe in men and women (Maudsley et al., 2009), but also in children (Hunter et al., 2005). However, it should be noted that Maudsley et al. (2009) found a laterality effect in gray matter only, while the present study reports from gray and white matter combined. In contrast, Nagae-Poetscher et al. (2004) and Pouwels and Frahm (1998) reported no significant asymmetry differences in the PFL. One explanation for the positive laterality results in the current study and Maudsley et al. (2009), versus the negative findings of Nagae-Poetscher et al. (2004) and Pouwels and Frahm (1998), could be increased power in

the former studies, both in terms of participant/session numbers and magnetic field strength that may slightly increase the signal to noise ratio.

Asymmetries in the PFL have previously been shown in both structural and functional (e.g. MR imaging studies (Specht, 2014)). When it comes to functional studies, asymmetries have been shown for language (Specht, 2014), mood states (Takeda et al., 2016), and during rest conditions (Bolduc et al., 2003; Tomasi and Volkow, 2012). The current study provides evidence of asymmetries in the PFL at the molecular level in bioenergetic properties, and one might speculate that this is related to other asymmetry findings. For example, differences in neuronal networks have been found between the hemispheres (Tomasi and Volkow, 2012). More specifically, Tomasi and Volkow (2012) found higher short- and long-range resting state connectivity in the left inferior and middle frontal gyrus as compared to homotopic areas in the right hemisphere, and these authors suggested that such asymmetries might relate to language functions in the left hemisphere. Two-thirds of the total cellular ATP production is spent on membranous Na^+/K^+-ion pump (Berndt and Holzhutter, 2013), and ATP production and demand is therefore especially high in regions of high synaptic density. Similarly, creatine and creatine kinase are located especially to regions of high energy production and/or demand (Kaldis et al., 1996; Rae, 2014). Thus, different network connectivity in the left and right hemisphere (Tomasi and Volkow, 2012) might result in different demands for energy supply and therefore an asymmetric distribution of creatine. The results should however be interpreted with caution as no measures of synaptic activity or ATP were made.

A leftward asymmetry was also observed for the metabolites NAA, Cho, and mI, but not for GLX (see Table S1). The results are partly consistent with a previous study (Maudsley et al., 2009), in which the authors found leftward frontal asymmetries for all three measured metabolites, NAA, creatine, and Cho, in gray matter, and for Cho also in white matter. The

asymmetries in the current study (gray and white matter combined) were found to be slightly higher as compared to Maudsley et al. (2009) for Creatine, Cho, and NAA.

Menstrual cycle effects in creatine asymmetry

As hypothesized, creatine levels were found to change across the menstrual cycle in the women, while remaining stable across testing points in men (see Figure 2). More specifically, the *asymmetry* of female creatine levels was found to change across the cycle phases. Left and right hemispheric creatine levels were significantly different in the menstrual and follicular phase, with particularly high leftward asymmetry in the follicular phase. In contrast, no hemispheric difference was found in the luteal phase. No previous study has investigated human brain creatine levels across the menstrual cycle. However, two studies investigated sex differences with inconsistent results. Although both studies found marginal sex differences in the frontal lobe, Chang et al (2009) suggested higher creatine levels in women as compared to men, while Hamakawa et al. (1999) found higher creatine levels in the right frontal lobe in men as compared to women. The current study could not replicate previous sex differences and found creatine asymmetry to fluctuate across the menstrual cycle. Thus, the study suggests that sex differences shown in previous studies are not invariant, but rather changes across the menstrual cycle with fluctuating sex-hormone levels (state dependent), and sex differences would partly depend on the cycle phase in which women are tested. In this regard the strength of the current study is particularly the finding of menstrual cycle related fluctuations in creatine as it might partly explain inconsistencies in the literature on sex differences in creatine.

Furthermore, the current study relates to the cycle effect of brain creatine metabolism that has previously been shown in other types of tissues (Somjen et al., 1991; Thompson et al., 2006; Zhu and Evans, 2001), and interaction of sex hormones and creatine in the rodent

brain (Allen et al., 2015). The mechanism for how sex hormones and creatine interact is largely unknown, however, Joncquel-Chevalier Curt et al. (2015) have proposed a preliminary model, according to which sex hormones interact with L-arginine:glycine amidinotransferase (AGAT) and sodium-dependent creatine transporter(SLC6A8). AGAT is an enzyme that catalyzes the transfer of an amidino group from L-arginine to glycine. The resultant guanidoacetic acid is the immediate precursor of creatine (Walker and Skorvaga, 1973). SLC6A8 is a creatine transporter that carries creatine across membranes, including the blood-brain barrier. Estradiol and testosterone is suggested to upregulate the local production of AGAT (Zhu and Evans, 2001), and testosterone also to upregulate SLC6A8. According to this model, the result of the current study might reflect sex-hormonal driven changes in creatine synthesis and/or creatine influx across the blood-brain barrier. (See below for discussions of different sex hormones).

The regional dependency, that is the change of creatine asymmetry across the menstrual cycle, primarily makes sense in relation to the well-established effect of sex hormones on brain organization (Neufang et al., 2009). The pattern of changing creatine asymmetries observed in the current study follows findings from studies addressing functional cerebral asymmetries across the menstrual cycle. Several studies have suggested stronger functional asymmetry in the follicular phase as compared to the menstrual phase (Cowell et al., 2011; Hjelmervik et al., 2012; Sanders and Wenmoth, 1998; Wadnerkar et al., 2008; but see Weis et al., 2008) or luteal phase (Alexander et al., 2002; Hjelmervik et al., 2012) in language asymmetry. The results of the current study also coincide with an imaging study that found menstrual cycle-related changing involvement of left and right PFL during a verbal working memory test (Joseph et al., 2012). Joseph et al. (2012), explained the changing asymmetry across the menstrual cycle with a change in interhemispheric inhibition (Hausmann and Güntürkün, 2000). The hypothesis was built on the assumption that a key mechanism in

generating functional asymmetry is the inhibition of the non-dominant hemisphere by the dominant one (Cook, 1984). For example it was shown by Weis et al. (2008) a reduced correlation between left and right IFG in the follicular phase as compared to menstrual phase during a verbal task. Whether the drop in right creatine levels is related to interhemispheric inhibition of left onto the right hemisphere presumes that creatine levels go hand in hand with neuronal activity. It should, however, be noted that the MRS measurement was conducted during rest and the extent to which fluctuations in creatine asymmetry are directly related to fluctuations in language lateralization is therefore unknown (but see Rango et al., 1997).

The role of estradiol, testosterone, and progesterone

Fluctuations in creatine asymmetries across the menstrual cycle in women (while stable in male controls) indicate that sex-hormones play a role. Higher gonadal hormone levels in general were found associated with leftward creatine asymmetry (high creatine in left PFL and/or low creatine in right PFL). In the cycle phase with strongest asymmetry – the follicular phase - only testosterone levels were significantly related to creatine asymmetry (see Figure 3; uncorrected for multiple comparisons). However, testosterone levels did not change significantly across the menstrual cycle in the current study. Therefore it is unlikely that cycle-related changes in creatine asymmetry were due to testosterone alone. Estradiol was a main candidate for modulating creatine levels (Joncquel-Chevalier Curt et al., 2015; Somjen et al., 1989; Somjen et al., 1991; Zhu and Evans, 2001), and although estradiol levels peaked in the follicular phase, we found only a small relationship to creatine. Whedon and Shorr (1957) reported that in two out of three cases, estradiol and testosterone in combination showed a larger increase in creatinuria (reflecting increased creatine synthesis) than testosterone administration alone, suggesting that it is maybe the combined effect of estradiol and testosterone that explains the increase in creatine asymmetry in the follicular phase in the current study.

An obvious question that arises is why the asymmetry was reduced in the luteal phase. The only hormone that was significantly different between the follicular and luteal phase is progesterone. However, progesterone levels alone were not found related to creatine symmetry. Instead, estradiol was found associated with leftward asymmetry. Estradiol and progesterone have previously been shown to interact (Baudry et al., 2013), also in relation to creatine (Allen et al., 2015), suggesting that neuroprotective effects of estradiol are antagonized by progesterone possibly through the reduction of estrogen receptors, or associated proteins or signaling pathways (Baudry et al., 2013). We therefore encourage future studies to include more participants in order to increase the power and/or allow for more advanced regression analyses that ideally also include non-linear relationships and interactions among the different hormones.

Implications

The traditional MRS methodology of using creatine as a standard control measure has been questioned (Rae, 2014), partly because creatine has been found to not be as stable as previously assumed, but rather vary with factors like diet (e.g. Dechent et al., 1999), and depressed vs. euthymic psychological state (Hamakawa et al., 1999). The findings of the current study are in line with this argument. The creatine asymmetry in women was found to fluctuate within relatively short time-periods across the menstrual cycle, suggesting that MRS studies should take sex and sex hormonal state into account when using creatine levels as a reference measure. It should be noted that men showed relatively stable creatine levels across three testing sessions and could arguably be used as a reference in male subjects. However, it should be tested whether varying testosterone levels in men (e.g. across the diurnal cycle) influence creatine concentration.

The findings of the current study also have clinical relevance since theories of impaired brain energy metabolism are central in our understanding of the pathology of psychiatric disorders (Allen, 2012; Stork and Renshaw, 2005). As a natural substance with fewer side effects, the potential of creatine as a therapeutic agent is being tested (e.g. in depression Allen et al., 2015). The understanding of how sex hormones and creatine interact is relevant to sex-sensitive treatments, since sex hormones might optimize or reduce the therapeutic effects of creatine supplements (Allen et al., 2015).

Menstrual cycle effect in choline

Exploratory analyses of remaining metabolites, showed a change in size of the resonance of Cho across the menstrual cycle (see Table S1 in supplementary material) depending on hemisphere. Cho concentration peaked in the left PFL in the follicular phase, accompanied with a drop in the right hemisphere. To our knowledge the finding of fluctuating Cho across the menstrual cycle is also novel. In animal litterature estradiol has been suggested to increase the synthesis of a precursor of choline, namely phosphatidylethanolamine-*N*-methyltransferase (Resseguie et al., 2007; Young, 1971). One previous brain MRS study in humans suggests Cho signal changes in the dorsolateral PFL in response to altered levels of ovarian hormones. More specifically, suppression of ovarian hormone production was assosiated with higher Cho level (Craig et al., 2007). The current study found Cho to be particularly high in the left PFL during the high estradiol follicular phase, but similar to Craig et al. (2007), low levels of Cho was found in the luteal phase with even higher levels of estradiol in addition to high levels of progesterone.

Cho is a measure of membrane metabolism or turnover, and changes in the Cho resonance could therefore reflect either increased membrane synthesis, or breakdown, or even changes in cell density (Rae et al, 2014). Alternatively, Cho could reflect levels of the

neurotransmitter acetylcholine. A study in rats (Wang et al., 2008) showed high correlations between Cho and acetylcholine in muliple brain regions, including frontally, and concluded that the Cho signal could be used as a measure of acetylcholine level in the brain. The cholinergic system modulates neural systems important for attention as well as learning and memory functions (Gold, 2003). Estradiol has been suggested to interact with the cholinergic system (Tinkler and Voytko, 2005); for example estrogen theraphy was found to prevent a decrease in cholinergic fibers in the prefrontal cortex in overectomized monkeys (Tinkler et al., 2004). The cholinergic system is also suggested to be asymmetric (Bianco and Wilson, 2009; Gutierrez-Ibanez et al., 2011; Hong et al., 2013). For example in parkinson patients, an acetylcholine inhibitor (blocking the reuptake of acetylcholine from the synaptic cleft) was found to increase frontal neuronal activity in the left hemisphere only and was assosiated with restored attentional cognitive functions (Possin et al., 2013). An asymmetric property of the cholinergic system might also relate to the asymmetric fluctuations of Cho across the menstrual cycle.

The changes in creatine and Cho across the menstrual cycle follows similar patterns and are most likely related to each other. Changes is creatine and Cho could either be explained by parallell processes or the change in one metabolite might facilitate the change in the other. We have previously suggested that gonadal hormones directly boost the production of a precursor to creatine. However, we cannot exclude the possibility that the increase in creatine production is a result of an sex-hormonal modulation of the cholinergic system which might in turn change the neuronal activity and the cellular energy demand.

Limitations

The current study was carefully controlled in terms of hormone measures and inclusion of a male control group. Still, some limitations need to be considered. As already mentioned, the sample size might be an issue when it comes to the detection of significant

correlations between hormones and creatine. The sample size also limits the number of regressors, and interaction effects among hormones were therefore not explored. Further, the voxels could arguably have been placed further lateral in order to cover more gray matter, and been limited to one functionally specific region (e.g. IFG).

Although voxel segmentation into tissue classes was performed using standard software (SPM8), we acknowledge that limitations of the segmentation method used may ultimately limit the accuracy of water-scaled concentration estimates (Gasparovic et al., 2006).

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Table S1. Mean and standard error for measured metabolites in women and men for all three cycle phases/male sessions and in total.

Women

	Menstrual		Follicular		Luteal		Total	
	L	R	L	R	L	R		R/L
Cre*^	5.44 ±.21	4.77 ±.21	5.84 ±.23	4.59 ±.25	5.32 ±.32	5.06 ±.24	5,17 ±.13	.88
NAA*	8.36 ±.30	7.41 ±.37	8.69 ±.24	7.28 ±.33	8.07 ±.28	7.50 ±.36	7,89 ±.19	.89
Cho*^	2.04 ±.09	1.82 ±.09	2.12 ±.11	1.70 ±.08	1.93 ±.10	1.87 ±.08	1,91 ±.05	.90
mI*	4.16 ±.25	3.50 ±.29	4.20 ±.26	3.60 ±.22	3.99 ±.33	3.98 ±.28	3,90 ±.15	.91
Glx	11.47 ±.82	12.12 ±.77	13.54 ±.83	13.10 ±.60	10.94 ±.79	13.13 ±.90	12,38 ±.39	1.09

Men

	Session 1		Session 2		Session 3		Total	
	L	R	L	R	L	R		R/L
Cre*	5.28 ±.20	4.89 ±.22	5.09 ±.22	4.77 ±.19	5.23 ±.19	4.58 ±.25	4,97 ±.13	.92
NAA*	8.40 ±.26	7.79 ±.39	8.36 ±.13	7.68 ±.34	8.47 ±.22	7.66 ±.31	8,08 ±.18	.92
Cho*	2.04 ±.07	1.80 ±.08	1.95 ±.08	1.81 ±.07	2.01 ±.08	1.80 ±.10	1,90 ±.06	.91
mI*	3.99 ±.28	4.3 ±.34	4.43 ±.41	3.64 ±.30	4.51 ±.43	3.78 ±.16	4,11 ±.23	.94
Glx	12.99 ±.75	12.67 ±.88	11.83 ±.91	12.17 ±.58	11.66 ±.79	11.96 ±.73	12,21 ±.40	1.01

Note: Table show mean and standard deviation for measured metabolites in women and men for all three cycle phases/male sessions and in total. For voxel GM content, the values in parenthesis refer to correlation coefficient of GM and Cre. Note that men are randomized into groups. R/L refer to right/left ratio across cycle phases/sessions and hemispheres. Abbreviations: Cre = Creatine; NAA = N-acetylaspartate; Cho = Choline; mI = Myo inositol; Glx = glutamate + glutamine; GM = gray matter. Repeated measures ANOVA with the factors Sex, female Cycle Phase/male Session, and Hemisphere showed a significant main effect of Hemisphere (indicated with *) in NAA ($F(1,27)=13.19$, $p=0.001$, $\eta^2=0.33$), Cho ($F(1,27)=15.6$, $p<0.001$, $\eta^2=0.37$), mI ($F(1,27)=7.73$, $p=0.01$, $\eta^2=0.22$). In Cho an significant interaction effect of Sex, Cycle Phase/Session, and Hemisphere (indicated with ^) was found ($F(2,54)=3.77$, $p=0.03$, $\eta^2=0.12$). Post hoc testing revealed that the interaction was driven by differences in Cho concentration between the female cycle phases. More specifically there was a difference between the left and right hemisphere in the follicular phase ($p<0.001$) only. It should also be noted that when women are not compared against the male control groups, a menstrual cycle effect (Cycle Phase x Hemisphere) is also found in Glx ($F=3.44$, $p=0.05$, $\eta^2=0.2$) driven by higher Glx in follicular phase than luteal phase in the left hemisphere ($p=.01$) and higher luteal Glx in right as compared to left hemisphere. For statistics for Cre and voxel GM content, see manuscript. The effect sizes (partial eta squared) reported in the table is from a lower level ANOVA conducted separately for women and men (interaction of Cycle Phase x Hemisphere in women, and Session x Hemisphere in men).

Table S2. Signal to noise ratio (SNR) and linewidth (LW) of female (n=15) and male (n=14) participants' spectra obtained from left (L) and right (R) prefrontal voxels are summarized in means ± standard deviation (parts per million (ppm)).

	Women		Men	
	L	R	L	R
SNR	13.8 ±2.84	13.69 ±3.04	14.48 ±3.31	14.1 ±2.98
LW	0.07 ±0.02	0.06 ±0.02	0.07 ±0.02	0.06 0.01

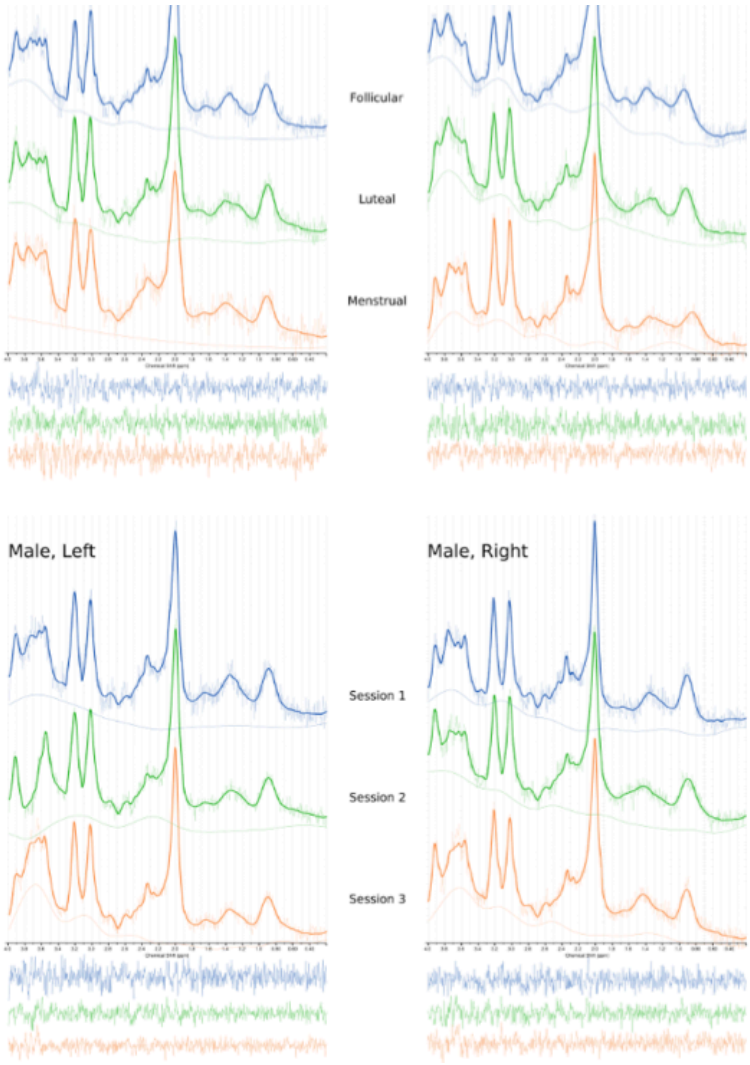

Figure S1. Spectra from three sessions in a female and a male participant acquired from right and left prefrontal lobe.