FROM SEX DIFFERENCES IN NEUROSCIENCE TO A NEUROSCIENCE OF SEX DIFFERENCES: NEW DIRECTIONS AND PERSPECTIVES

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FROM SEX DIFFERENCES IN NEUROSCIENCE TO A NEUROSCIENCE OF SEX DIFFERENCES: NEW DIRECTIONS AND PERSPECTIVES

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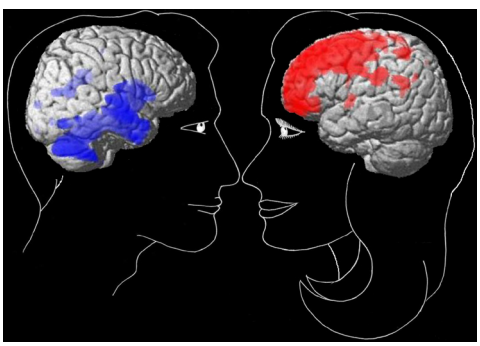

Sex differences in brain structure.
Areas with significantly larger regional gray matter volumes in men are depicted in blue, areas with significantly larger regional gray matter volumes in women in red. Original data acquired by Belinda Pletzer.

This research topic aims to integrate scattered findings on sex differences in neuroscience into a broader theory of how the human brain is shaped by sex and sex hormones in order to cause the great variety of sex differences that are commonly observed. It can be assumed that these differences didn't occur arbitrarily, but that they rather determined and still determine evolutionary success of individuals and were shaped by the processes of natural and in particular sexual selection. Therefore, sex differences are not negligible and sex difference research cannot be discriminating against one sex or the other. In fact a better understanding of the underlying causes of sex differences has great advantages for both men and women and society as a whole, not only in terms of health care, but in every aspect of life. Gender equality can only work out if it is equally well understood for men and women what their individual resources and needs are. Therefore, it is of great importance to pave the way for identifying the underlying principles of structural and functional brain organization that cause men and women to act, think and feel differently.

To this end it is of particular interest to identify possible similarities and interrelations between sex differences that did so far stand separately, in order to investigate whether they share a common source. To understand, where a specific sex difference comes from and whether or not it is caused by the same principle as other sex differences, it is necessary to explicitly link sex differences in behavior to their neuronal correlates and vice versa link sex differences in brain structure and function to their behavioral outcomes. In particular a new understanding of male and female brain functioning may arise from findings on how sex hormones interact with various neurotransmitter systems.

In the past few years several findings demonstrated that women's behavior is influenced by the sex hormone fluctuations they experience naturally during their menstrual cycle to the extent that

sex differences may only be detectable in one cycle phase but not another. The study of menstrual cycle dependent effects gives important hints about which sex differences are activational and which are organizational. Additionally it only recently came to attention, that hormonal contraception may alter a women's mood, cognition and behavior as a consequence of changes in brain structure and function. The underlying mechanisms are so poorly understood that it is even hard to predict, whether hormonal contraception will mask or amplify sex differences in a given task. Since the oral hormonal contraceptive pill is meanwhile used by 100 million women worldwide and even by teenagers whose brains are not yet fully developed, the question of how the synthetic steroids contained in hormonal contraceptives act on the brain is to be studied hand in hand with naturally occurring sex differences.

This topic summarizes the current state of the art in sex difference research and gives new perspectives in terms of hypothesis generation an methodology. Both are necessary to gain a complete picture of what it is that makes a brain male or female and move towards a neuroscience of sex differences.

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EDITORIAL
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Editorial: From sex differences in neuroscience to a neuroscience of sex differences: new directions and perspectives

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Keywords: sex differences, sex hormones, menstrual cycle, hormonal contraception, menopause, neurotransmitters, stress hormones, sex role

While we are looking back at a century of behavioral research on sex differences in cognition and emotion, sex differences have for a long time been disregarded as a confound in neuroscience.

Several researchers still argue that sex differences in cognition are overall small and negligible (Hyde and Linn, 1988, 2006; Hyde, 2005, 2006). Indeed the data so far are by no means consistent. These inconsistencies are to a large part attributable to small sample sizes and low power on the one hand and a large variation in methodologies on the other hand. This is even more true for studies on menstrual cycle and hormonal contraceptive dependent effects on the brain, as will be pointed out in this Research Topic (Pletzer and Kerschbaum, 2014; Sundström Poromaa and Gingnell, 2014).

However, another reason that these inconsistencies still exist may also be that neuroscience, looking at the topic from the angle of political correctness, has refrained from studying sex differences in the brain in more detail. One major goal of this Research Topic is to change this view and look at the topic from a different angle:

Women don't have to be like men to be treated as equal. Women have a right to be women and if that includes being different from men, women have a right to be different from men and—importantly—also vice versa. After all, innovation stems from diversity (e.g., Hewlett et al., 2013). Furthermore, women have a right to understand these differences as well as the hormonal changes that not only their body, but also their brain, goes through during their lifetime. So it's important that neuroscience starts paying more attention!

The aim of this Research Topic is to point out directions and perspectives on how to resolve inconsistencies in sex difference research. The idea is to move from scattered findings on sex differences in the brain to a neuroscience of sex differences that will help researchers to understand and predict sex differences in their findings and integrate them into their theories.

From the beginning of sex difference research, it has been hypothesized that sex differences in behavior are at least in part driven by hormonal influences on the brain either during development (organizational) or later in life (activational) (e.g., Kelly et al., 1999). Of course, not all sex differences can be attributable to sex hormones, but genetic, epigenetic, and chromosomal effects also play an important role, as pointed out by Ivanka Savic' contribution to this topic (Savic, 2014). However, sex hormone influences have been hypothesized to explain that variation in performance is higher in women than in men (e.g., Hausmann and Güntürkün, 1999). Therefore, sex hormones are of special interest to this topic as one important factor that might explain inconsistencies between sex difference studies.

Accordingly, the idea for this Research Topic was born during a study on sex differences in number processing. In a number bisection task, we found for two behavioral effect that sex differences in brain activation patterns were present during one cycle phase (follicular or luteal), but

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not during the other (Pletzer et al., 2011). In a follow-up study using the same task we furthermore recognized that women using hormonal contraceptives differed from naturally cycling women in the same way as men (Pletzer et al., 2014). These findings have important methodological implications for research on sex differences.

First, I propose here, that sex differences should always be investigated by comparing men to naturally cycling women, i.e., naturally occurring sex differences. The question of hormonal contraceptive dependent influences on brain and behavior is related, but separate.

Given that in developed countries about half of all women below the age of 45 rely on some method of hormonal contraception (Guttmacher Institute, 2015), hormonal contraception may represent a major confound in the study of sex differences. Only a handful of researchers have so far investigated hormonal contraceptive dependent effects on cognition, brain structure or brain function and only few consistencies arise. Within this topic, these findings are reviewed, potential mechanisms of action of hormonal contraceptives on brain and behavior are discussed and perspectives for research on hormonal contraceptive dependent effects are suggested (Pletzer and Kerschbaum, 2014).

Second, I argue that differences between men and naturally cycling women cannot be investigated without taking menstrual cycle phase into account. Despite an early interest in menstrual cycle dependent influences on mood (Frank, 1931), menstrual cycle dependent changes in cognitive functions have only been investigated for about 25 years now, starting with a pioneer study by Hampson (1990a,b). Only few studies have however investigated menstrual cycle dependent changes using neuroimaging methods. In this topic, menstrual cycle dependent changes in brain and behavior were carefully reviewed by Sundström Poromaa and Gingnell (2014). However, here again, results on the direction of changes as well as on the particular hormones responsible for the changes are inconsistent and more detailed studies are lacking. Possible reasons include that menstrual cycle research is costly and time consuming. This is due to difficulties in the recruitment of women, who do not use hormonal contraceptives, on the one hand, and large drop-outs on the other hand due to inaccurate self-reports of menstrual cycle phase. In order to clearly differentiate between the effects of estradiol and progesterone on brain functions, it is of uttermost importance to test in clearly defined and rather narrow time windows of a woman's individual menstrual cycle and ideally test at three different time-points: menses, pre-ovulation, and mid-luteal. Only few researchers have so far realized such a design (e.g., Weis et al., 2008; De Bondt et al., 2015). Within this Research Topic, one contribution presents for the first time neuroimaging data of a naturally cycling woman accompanied over four menstrual cycles (Arélin et al., 2015).

Other exploratory approaches include a comparison of high (mid-luteal) and low (early follicular) phases in within- as well as between-subjects designs. Both approaches have been successfully realized within this Research Topic in order to explore menstrual cycle dependent effects on reward processing and decision making (Derntl et al., 2014; Reimers et al., 2014).

However, especially changes in brain function and behavior due to the pre-ovulatory estradiol peak have recently come to attention (Jacobs and D'Esposito, 2011). From an evolutionary perspective, such pre-ovulatory changes are of particular interest for sexual selection. Jacobs found, that the pre-ovulatory estradiol peak particularly influences dopamine-dependent cognitive functions. Behavioral and methodological implications of this estradiol-dopamine interaction have been outlined within this topic by Colzato and Hommel (2014). Examining the interaction between sex hormones and relevant neurotransmitter systems is one important step toward a neuroscience of sex differences. By combining the knowledge from cellular, molecular, and animal research on how sex hormones interact with neurotransmitter systems with the knowledge of which cognitive functions these neurotransmitters contribute to, we can formulate clear hypotheses on how sex hormones might influence a certain behavior. Therefore, this topic includes an extensive review on such sex hormone-neurotransmitter interactions (Barth et al., 2015). Such interactive approaches would also allow us to reduce the costly and complex menstrual cycle designs outlined above to those phases where the hypothesized interactions are to be expected.

The same argumentation can be applied to the interaction between sex hormones and other hormone systems. Sex and stress hormone interactions (Kirschbaum et al., 1992; Andreano et al., 2008) have especially drawn attention due to their potential role for the vulnerability to mental health disorders, such as depression. Two articles within this Research Topic focus on this problematic, focusing on both the organizational effects (Goldstein et al., 2014) and the activational effects (Gobinath et al., 2014) of sex hormones. Furthermore, the article of Sorwell and colleagues within this topic addresses sex hormone interactions with the anti-stress hormone DHEA (Sorwell et al., 2014).

Importantly, in both domains—sex hormone neurotransmitter interactions (Barth et al., 2015), and sex hormone—stress hormone interactions (Gobinath et al., 2014; Goldstein et al., 2014)—the articles are organized along hormonal transition periods across the female life-span. Everything outlined so far implicitly referred to sex differences between men and women in their reproductive years. However, periods of much more extreme hormonal changes than during a normal menstrual cycle, such as puberty, pregnancy, and menopause, may provide us with important insights on how sex hormones affect a woman's brain and behavior. Therefore, post-menopausal functional changes have been reviewed separately within this topic (Comasco et al., 2014).

Thus, we have extensively captured potential variations in brain function and behavior due to hormonal status in the female group focusing mainly on the sex hormones estradiol and progesterone. However, several sex differences have early on been attributed to the organizational and activational effects of testosterone, particularly in the male group. Therefore, when studying sex hormone modulation of sex differences, testosterone levels should also be taken into account. Within this topic, testosterone actions on the brain have been reviewed by Celec et al. (2015). Testosterone and its metabolites also receive

particular attention in the articles contributed by Sorwell et al. (2014), as well as Krajnik et al. (2014).

Summing up, sex difference research should profit in the future methodologically from taking into account the hormonal status of participants, especially in the female group and theoretically from more straightforward research questions and clearly formulated hypotheses by taking into account for example the interactions between sex hormones and neurotransmitter systems as well as other hormone systems. Here, I also propose that one direction sex difference research should aim at in the future is more integrative approaches, explaining sex differences across a variety of behaviors. In one of my own articles I outline the idea that sex differences might stem from a common principle in brain organization, i.e., the lateralization of brain functions (Pletzer, 2014). Sex differences in hemispheric asymmetries have long attracted attention and are also in the focus of Savic's contribution to this Research Topic (Savic, 2014). My article thereby focuses less on particular abilities than on cognitive strategies in men and women (Pletzer, 2014).

Furthermore, in our latest contribution to the topic we try to pick up the theme introduced in the beginning of this Editorial. How strong are sex differences really and are they worth investigating? One argument against sex difference research has always been that the variation within men or women is much larger than the differences between men and women. As outlined in the previous paragraphs there are quite a number of ways how sex hormones, might contribute to this within-group variation. However, it might also be interesting for researchers to capture this variation within the male and female groups. We therefore dedicated our latest contribution to the topic of sex role orientation and the question how we can assess and which factors affect the individual maleness or femaleness of a person (Pletzer et al., 2015). Because, in the end, sex may be one important factor influencing our behavior, but not only men and women differ. Every individual is different and should be accepted as such.

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50 years of hormonal contraception—time to find out, what it does to our brain

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Hormonal contraceptives are on the market for more than 50 years and used by 100 million women worldwide. However, while endogenous steroids have been convincingly associated with change in brain structure, function and cognitive performance, the effects of synthetic steroids contained in hormonal contraceptives on brain and cognition have barely been investigated. In this article we summarize the sparse findings, describing brain structural, functional and behavioral findings from the literature and suggest that synthetic steroids may contribute to masculinizing as well as feminizing effects on brain and behavior. We try to identify methodological challenges, explain, how results on endogenous steroids may transfer into research on hormonal contraceptives and point out factors that need to be controlled in the study of hormonal contraceptive dependent effects. We conclude that there is a strong need for more systematic studies, especially on brain structural, functional and cognitive changes due to hormonal contraceptive use. The hormonal contraceptive pill is the major tool for population control. Hence, such behavioral changes could cause a shift in society dynamics and should not stay unattended.

Keywords: hormonal contraceptives, synthetic steroids, progestins, androgenicity, ethinyl estradiol

INTRODUCTION

Hormonal contraceptive pills are on the market for more than 50 years (see **Box 1**). Today, about 100 million women worldwide use combined oral contraceptives (Petitti, 2003). However, despite the well characterized impact of endogenous female sex hormones on structure and physiology of the brain and their impact on cognition and mood, very little is known about the impact of synthetic steroids on cognitive abilities, brain structure and function. The impact of hormonal contraceptives on the brain is of great importance equally to the private user and physicians as well as to clinical and basic research. The far reaching consequences of hormonal contraceptives are illustrated by a few contrasting facts. (i) Kinsley and Meyer notice that the intake of steroids by an athlete is considered as abuse and "doping" by androgens is strongly sanctioned by society. On the contrary the intake of steroids in women is recommended for birth control around the world (http://www.scientificamerican.com). (ii) Adolescent girls start taking hormonal contraceptives earlier and earlier, often shortly after onset of puberty (Parkes et al., 2009). However, the majority of research on steroid actions in the brain focuses on post-menopausal hormone replacement therapy. (iii) Traditionally, medical as well as psychological research focused on male participants, because hormone fluctuations throughout the menstrual cycle were suspected to affect the results—rightly, as it turned out. Nowadays, numerous women participate in scientific studies. However, while participants on medication are excluded, studies hardly control for the use of hormonal contraception.

In this article we summarize the sparse findings indicating brain structural, functional and behavioral changes due to hormonal contraceptive use and try to give methodological impulses for future research. The interpretation of these changes are demanding and at the moment speculative. Although, endogenous female sex hormones have been convincingly associated with changes in brain structure and physiology—estradiol and progesterone modulate e.g., glutamatergic, GABAergic, serotonergic and dopaminergic transmission (see Sacher et al., this topic)—the neuronal targets of synthetic steroids are almost unknown. Changes in brain structure and chemistry cause changes in cognition, emotion and personality and consequently in observable behaviors. If a majority of women use hormonal contraception, such behavioral changes could cause a shift in society dynamics. Since the pill is the major tool for population control, it is time to find out what it does to our brain.

EFFECTS OF HORMONAL CONTRACEPTIVES: "FEMINIZING" OR "MASCULINIZING"?

The combined oral contraceptive pill (OC) typically contains 0.02–0.04 mg ethinylestradiol and varying levels of synthetic progestins, which can be classified by their generation. Progestins are a heterogenous class of synthetic steroids, which differ in their strength of binding to steroid receptors, the sex hormone binding globuline or to enzymes involved in the metabolism of endogenous steroids (e.g., Sitruk-Ware, 2006). Furthermore, they differ in their impact on blood glucose levels and the

Box 1 | A short history of hormonal contraception.

Within about a century, physicians, biologists, and chemists around the world elucidated the physiology of the ovary, manipulated its function, and triggered a global experiment, which influenced our society. At the end of the 19th century, anatomists concluded from histological sections that the corpus luteum in the ovary is a gland secreting a factor into the blood relevant to pregnancy (Frobenius and Fraenkel, 1999). In the early 1920s, the Austrian physician, Ludwig Haberlandt (1885–1932), published his findings on temporary sterilization of the female body. He realized that transplantation of ovaries from pregnant to non-pregnant animals prohibits pregnancy in the receiver. In a translational approach, Haberlandt suggested hormonal contraception also in women and collaborated with the company of G. Richter in Budapest (Hungary) to develop contraceptives for women (Haberlandt, 2009). In 1923, Edgar Allen and Edward Adelbert Doisy (1893–1986) published a bioassay, which used the vaginal epithelium response in castrated rodents to identify the bioactive component among corpus luteum factors as well as other sex hormones (Frobenius and Fraenkel, 1999). In the 1930s, progesterone was isolated from glandular extracts independently by four research groups, and its chemical structure was characterized by the chemist Karl Heinrich Slotta (1895–1987) (Frobenius and Fraenkel, 1999; Hawgood, 2001). In 1944, the Germans, Bickenbach und Paulikovics, published that progesterone (20 mg, daily) suppresses ovulation in women (Ludwig, 2011). Because progesterone is metabolized during the entero-hepatical passage, synthesis of chemically modified gestagens was a major challenge. Carl Djerassi, an Austrian-American chemist (born in Vienna in 1923), synthesized norethindrone, the first oral contraceptive, in the early 1950s. In the late 1950s, the American biologist, Gregory Goodwin Pincus (1903–1967) proved in a large scale study that oral application of hormonal contraceptives prevents pregnancy in women. In 1960, the "Food and Drug Administration" in the USA legalized the first contraceptive (Enovid®), a combination pill containing 10 mg of the progestogen, norethynodrel, and 150 μg of the estrogen, mestranol. In 1961, the pill (Anovlar®, Bayer Schering Pharma AG) was legalized in Europe. The minipil, a progestine-only pill, was introduced into the market in the 1960s.

lipid profile (e.g., Sitruk-Ware, 2006). Importantly, older generation progestins (e.g., Levonorgestrel, desogestel, gestoden, norgestimat), are as derivates of 19-nortestosterone, a common anabolic steroid (Brueggemeier, 2006), able to activate androgen receptors and exert androgenic actions (Sitruk-Ware, 2006; Wiegratz and Kuhl, 2006). Newer progestins (e.g., dienogest, drospirenone) on the other hand, bind very specifically to the progesterone receptor and are anti-androgenic (Wiegratz and Kuhl, 2006).

In the following we describe several mechanisms that may explain the effects of synthetic steroids on brain and behavior. We refer to an effect as "*feminizing*," if it enhances a structural or functional sexual dimorphism favoring women. We refer to an effect as "*masculinizing*," if it reduces a structural or functional sexual dimorphism favoring men.

SYNTHETIC STEROIDS—POSSIBLE MECHANISMS OF ACTION

Since ethinylestradiol and synthetic progestins act on estrogen and progesterone receptors and OC reduce endogenous testosterone levels (e.g., Jung-Hoffman and Kuhl, 1987; Graham et al., 2007; Hietala et al., 2007), any hormonal contraceptive, irrespective of the progestin component, may show feminizing effects on brain and behavior.

However, hormonal contraceptives do also lead to a reduction of endogenous estradiol and progesterone levels (e.g., Sahlberg et al., 1987). In the presence of high levels of progesterone, testosterone-actions are impaired, because progesterone has a high affinity for the enzyme 5α-reductase, which is responsible for the conversion of testosterone into the physiologically more active dihydrotestosterone (Wright et al., 1983). If progesterone levels are reduced, more testosterone can be converted to dihydrotestosterone. Thus, any hormonal contraceptives, irrespective of the progestin component, may facilitate testosterone actions on the brain, thereby masculinizing brain structure, function and behavior. Alternatively, it has been argued that some masculinizing effects are promoted by estrogen receptors after testosterone has been locally converted to estrogen via the enzyme aromatase (Roselli, 2007). Consequently, estrogenic actions of ethinylestradiol may contribute to possible masculinizing effects of hormonal contraceptives on the brain.

ANDROGENIC vs. ANTI-ANDROGENIC PROGESTINS

Furthermore, the actions of synthetic progestins on the androgen receptors, may contribute to possible masculinizing and feminizing effects. While androgenic progestins may promote masculinizing effects, anti-androgenic progestins may promote feminizing effects. This distinction has important methodological consequences. The preferable approach to studying the effect of hormonal contraceptives in brain and behavior is a within-subjects design, i.e., comparing women to themselves, before, during and after the period of contraceptive use. Since this is however complicated, costly and time-consuming, hormonal contraceptive dependent effects are often studied by comparing a group of OC users to a group of non-users. If the group of OC users is heterogenous with respect to the androgenicity of the progestin component, possible feminizing and masculinizing effects may counteract each other, leading to results that either under- or over-estimate the actual effects and are hard to compare between different studies. While the first studies in this domain added important explorative impulses, by demonstrating that OCs may indeed influence brain and behavior, we strongly argue that the androgenicity of the progestin component is an important factor that should be controlled in future studies. An interesting observation from our own research experience in that respect may help when comparing findings between studies. We conducted two studies on the effects of OCs only about a year and a half apart, the first one in middle Europe (Austria; Pletzer et al., 2014a), the second one in the US (California, Pletzer et al., 2014b). While in the Austrian sample (Pletzer et al., 2014a), the group of OC users consisted of a majority of women using newer pills containing anti-androgenic progestins, in the US sample (Pletzer et al., 2014b), all OC users were on older pills containing androgenic progestins.

EFFECTS OF HORMONAL CONTRACEPTIVES ON BRAIN STRUCTURE

Adult brain structure is not static, but subject to dynamic changes with age. These changes do differentially affect different brain areas, i.e., gray matter volumes in some areas decline more strongly with age than others. A strong age-related decline has for example been demonstrated in the prefrontal cortex, as well as the hippocampus (e.g., Sowell et al., 2003).

Recent results demonstrated that regional gray matter volumes in the prefrontal cortex, as well as the anterior cingulate gyrus are larger in mixed samples of androgenic and anti-androgenic OC users compared to non-users (Pletzer et al., 2010; DeBondt et al., 2013). These regions are already larger in women compared to men (e.g., Good et al., 2001; Pletzer et al., 2010). However, regional gray matter volumes of OC-users were also larger in the cerebellum, hippocampi, parahippocampal and fusiform gyri (Pletzer et al., 2010; DeBondt et al., 2013). Those regions are on the average larger in men compared to women (e.g., Good et al., 2001; Pletzer et al., 2010). Results from rodent hippocampi suggest that these volume increases may be attributed to an increase in synaptic spine density mediated by estrogen receptors (e.g., Murphy et al., 1998; McEwen, 2002; Smith et al., 2009), but an increase in astrocyte volume in response to estradiol has also been suggested (e.g., Spencer et al., 2008).

Newer results from our own lab, suggest that the effects in cerebellum, parahippocampus and fusiform gyri are attributable to OCs containing anti-androgenic progestins, while the results in the prefrontal cortex are to be interpreted with care (Pletzer et al., submitted). We demonstrate that some structural effects increase with the duration of pill use, some interact with the age of the participants and some may not be completely reversible, and hence the duration of previous pill use in the group of non-users plays an important role. Thus, despite the androgenicity of progestins, the duration of (previous) pill use and age are important factors, that should be controlled when studying the effects of OCs on brain and behavior. OCs, in particular those containing anti-androgenic progestins, are commonly used by younger women, while naturally cycling women tend to be on average a few years older.

EFFECTS OF HORMONAL CONTRACEPTIVES ON BRAIN FUNCTION—NEUROCOGNITIVE FINDINGS

Synthetic steroids may however, not only cause a structural reorganization of the brain, but—even more importantly—induce changes in neurochemistry and brain function, which are currently relatively unexplored. Despite the relatively robust finding that OC users show altered mate preferences (e.g., Alvergne and Lummaa, 2009), accompanied by changes in brain activation patterns during viewing of erotic stimuli (Abler et al., 2013), OC dependent changes in cognitive performance have not been studied systematically.

Scattered over several countries and decades, it has been reported that OC users show enhanced verbal memory (Mordecai et al., 2008), recognition working memory during sleep deprivation (Wright and Badia, 1999), a lack of memory impairment due to cortisol (Kuhlmann and Wolf, 2005) and better dream recall (Sheldrake and Cormack, 1976) compared to non-users. These verbal abilities and memory are usually thought to favor women (e.g., Andreano and Cahill, 2009). However, non-significant effects were reported for other measures of verbal abilities, like verbal fluency (Mordecai et al., 2008) or a verbose-quential task (Gordon and Lee, 1993), while verbal reaction times were slower in OC users compared to non-users in an older study (Garrett and Elder, 1984). First evidence for brain functional differences between OC users and non-users has also been reported in the verbal domain. A German brain imaging study (Rumberg et al., 2010) observed that during a word generation task OC users showed stronger activations in right-hemispheric task-specific areas than non-users. This is of particular interest, since the peak coordinates of the task-specific activations were left-lateralized in all participants and sex differences in lateralization have long been discussed in particular with respect to verbal tasks (e.g., Renteria, 2012).

On the other hand, some studies report better mental rotation performance in OC users compared to non-users (Wright and Badia, 1999; Wharton et al., 2008), although other studies report non-significant effects on visuospatial tasks (Gordon and Lee, 1993; Mordecai et al., 2008). Spatial abilities have robustly been observed to favor men (Andreano and Cahill, 2009). Wharton et al. (2008) nicely demonstrated that mental rotation performance does not only correlate with hormonal contraceptive use, but also with the androgenicity of the progestin component. Users of drospirenone-containing contraceptives performed worse on the mental rotation task than non-users. Neuroimaging studies exploring functional differences in spatial tasks between OC-users and non-users are still lacking. However, it has been demonstrated that brain activation patterns are masculinized in numerical tasks (Pletzer et al., 2014a), which have been related to spatial abilities (e.g., Hubbard et al., 2005).

Furthermore, OC users perform like men in an emotional memory paradigm, designed by Cahill and coworkers (Nielsen et al., 2011, 2013) and in a Navon paradigm (Pletzer et al., 2014b), both studies conducted on US samples. Brain functional differences between OC users and non-users have also been reported during the resting state (Petersen et al., 2014), during face processing (Mareckova et al., 2012) and during reward processing (Bonenberger et al., 2013). However, further more systematic studies are needed to reveal the true nature of OC-dependent effects on cognition as well as the impact of synthetic steroids on the neuronal correlates. Importantly, it has been demonstrated that endogenous sex hormones, in particular estrogen, affect cognitive performance differentially in different subpopulations of women, as some neurotransmitters (e.g., dopamine) affect behavior in an inverse U-shaped manner (Jacobs and D'Esposito, 2011; Colzato and Hommel, 2014). Hence, like in the study of menstrual cycle dependent effects (Colzato and Hommel, 2014), it might be worth considering neurotransmitter baseline levels, when studying the effects of hormonal contraceptives on brain and behavior.

EFFECTS OF HORMONAL CONTRACEPTIVES ON EMOTION

Reports on OC-related mood changes are inconsistent, ranging from beneficial in most women (Oinonen and Mazmanian, 2002) to increased rates of depression, anxiety, fatigue, neurotic symptoms, compulsion and anger (Robinson et al., 2004;

Kulkarni, 2007). Repeated findings of beneficial mood changes may however be biased by data sampling. Only women, who continue the intake of oral contraceptives ("survivor effect") are included in those studies, while women, who discontinue the use of oral contraceptives due to negative emotional side effects, do not contribute to these results (Oinonen and Mazmanian, 2002).

Possible physiological mechanisms underlying both positive and negative mood swings in oral contraceptive users are manifold and at the moment speculative. Elevated levels of estradiol have anti-depressive effects (Moses-Kolko, 2009; Estrada-Camarena, 2010), presumably due to its serotonin (5-HT) enhancing property (e.g., Bethea et al., 2002). A decline in estradiol at the end of the menstrual cycle, post-partum or in the menopause has been associated with negative mood changes and depressive symptoms during these phases (Moses-Kolko, 2009). On the one hand combined OCs contain ethinyl-estradiol as synthetic agonist for estradiol receptors, which could promote positive mood changes. Antagonistic properties for the 5-HT3 receptor have been demonstrated not only for estradiol, but also for ethinylestradiol (Wetzel et al., 1998). On the other hand the levels of endogenous estradiol decline as a consequence of OCs (e.g., Sahlberg et al., 1987), which could result in negative mood changes. In female cynomolgus monkeys on OCs a decreased prolactin response was observed, suggesting reduced serotonergic activity (Henderson and Shively, 2004). Progesterone, however, may promote positive mood changes at low concentrations and negative mood changes at high concentrations due to biphasic effects on GABAergic neurons (Andréen et al., 2009). Again, synthetic progestins as contained in OCs simultaneously act as progesterone receptor agonist and reduce the level of endogenous progesterone (Wright et al., 1983; Sahlberg et al., 1987). In a meta-analysis Oinonen and Mazmanian (2002) suggest that the progesterone/estrogen ratio correlates to the direction of emotional changes.

In summary, these results suggest that there are (at least) two populations of women with differential emotional responses to oral contraceptive use. Interestingly, the majority of studies focused on depressive symptoms, while other emotional and personality dimensions, like aggression or empathy, have hardly been investigated.

PUBERTY AND HORMONAL CONTRACEPTIVES

Progestin affecting either metabolism of neurosteroids or binding to $GABA_A$ receptors may have not only transient but also neuroplastic consequences. We assume that the potential influence of progestins on GABAergic transmission is highly relevant in pubescent girls using hormonal contraceptives. (1) With onset of puberty, neurosteroid sensitive GABA receptor expression increases at extrasynaptic sites in female mice (Smith, 2009). (2) Enhanced GABAergic transmission shortens whereas depression of GABAergic transmission extends the critical period in structural consolidation of neuronal circuits in the visual cortex in mice (Hensch, 2005). (3) Final volume of the prefrontal cortex is not reached until the early twenties in humans (Yurgelun-Todd, 2007). The maturing of the prefrontal cortex is associated with improvement in cognitive abilities as well as behavioral control (Yurgelun-Todd, 2007). The prefrontal cortex appears to be one target of structural changes in hormonal contraceptive users (Pletzer et al., 2010; DeBondt et al., 2013). Accordingly, pharmacological intervention by the early use of hormonal contraceptives could affect the differentiation of neural circuits in the prefrontal cortex.

SUMMARY AND CONCLUSION

First and foremost, we conclude that there is a strong demand for additional studies on how hormonal contraceptives affect the brain from the molecular to the behavioral level. Thus, future studies aiming to investigate "normal" brain functioning, should control for the use of hormonal contraceptives among their participants. At both the structural as well as behavioral level, feminizing and masculinizing effects of hormonal contraceptives have been observed simultaneously. However, these changes may show differential manifestation at the behavioral level in different subpopulations of women. As the number of women using oral contraceptives constantly increases, while the age of first contraceptive use constantly decreases down to sensitive neuroplastic periods during puberty, the associated changes in personality and social behavior imply significant consequences for society.

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Menstrual cycle influence on cognitive function and emotion processing—from a reproductive perspective

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The menstrual cycle has attracted research interest ever since the 1930s. For many researchers the menstrual cycle is an excellent model of ovarian steroid influence on emotion, behavior, and cognition. Over the past years methodological improvements in menstrual cycle studies have been noted, and this review summarizes the findings of methodologically sound menstrual cycle studies in healthy women. Whereas the predominant hypotheses of the cognitive field state that sexually dimorphic cognitive skills that favor men are improved during menstrual cycle phases with low estrogen and that cognitive skills that favor women are improved during cycle phases with increased estrogen and/or progesterone, this review has not found sufficient evidence to support any of these hypotheses. Mental rotation has gained specific interest in this aspect, but a meta-analysis yielded a standardized mean difference in error rate of 1.61 (95% CI −0.35 to 3.57), suggesting, at present, no favor of an early follicular phase improvement in mental rotation performance. Besides the sexually dimorphic cognitive skills, studies exploring menstrual cycle effects on tasks that probe prefrontal cortex function, for instance verbal or spatial working memory, have also been reviewed. While studies thus far are few, results at hand suggest improved performance at times of high estradiol levels. Menstrual cycle studies on emotional processing, on the other hand, tap into the emotional disorders of the luteal phase, and may be of relevance for women with premenstrual disorders. Although evidence at present is limited, it is suggested that emotion recognition, consolidation of emotional memories, and fear extinction is modulated by the menstrual cycle in women. With the use of functional magnetic resonance imaging, several studies report changes in brain reactivity across the menstrual cycle, most notably increased amygdala reactivity in the luteal phase. Thus, to the extent that behavioral changes have been demonstrated over the course of the menstrual cycle, the best evidence suggests that differences in sexually dimorphic tasks are small and difficult to replicate. However, emotion-related changes are more consistently found, and are better associated with progesterone than with estradiol such that high progesterone levels are associated with increased amygdala reactivity and increased emotional memory.

Keywords: menstrual cycle, estradiol, progesterone, cognition, emotion, functional magnetic resonance imaging

INTRODUCTION

The menstrual cycle has attracted research interest ever since the 1930s (Frank, 1931). Despite the extensive research on this excellent and ecological model of ovarian steroid influence on emotion, behavior, and cognition, relatively few findings have emerged as conclusive. In fact, already in 1973 did Barbara Sommer review the existing literature (at that point 33 scientific papers were available) and concluded that no menstrual cycle-related changes in cognitive and perceptual-motor performance were evident (Sommer, 1973). Yet, she also concluded that methodological problems were common, specifically concerning menstrual cycle definition and hormonal state. With increasingly accessible methods for steroid hormone analyses, both in serum and saliva, tremendous improvements in menstrual cycle studies have been achieved over the past years. One example of this is premenstrual dysphoric disorder (PMDD), which used to be a loosely defined syndrome with numerous, but inadequate, treatment options ranging from herbal remedies, to vitamins and progestagens. With strict definitions in the Diagnostic and Statistical Manual of Mental Disorders, thorough menstrual cycle phase staging and high-quality randomized clinical trials, clinicians today are able to offer afflicted women effective and evidence-based treatments (Marjoribanks et al., 2013).

The idealized menstrual cycle consists of 28 days, but it is normal that cycle length varies between 21 and 35 days (Lenton et al., 1984a). The menstrual cycle length decreases with advancing age (Lenton et al., 1984a), and approximately 7% of menstrual cycles are shorter than 26 days (Brodin et al., 2008). Oligomenorrhea is defined as menstrual cycle length of 35 days or more (Treloar et al., 1967; Chiazze et al., 1968), and is in turn one of the

criteria for the polycystic ovary syndrome (PCOS) (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004). In an infertility setting, which may not be entirely representative of the general population, 5.4% of menstrual cycles are 35 days or longer (Brodin et al., 2008). The follicular phase is characterized by follicular development, in response to increased levels of follicle stimulating hormone (FSH) in the early follicular phase, but later, as a dominant follicle has been selected the stimulatory need for FSH gradually diminishes. The pool of growing follicles progressively increase their estradiol production, but the pre-ovulatory estradiol surge is, in fact, a direct signal from the dominant follicle to the hypothalamus that it is ready for the final events leading up to ovulation (Speroff and Fritz, 2010). Typically, the first seven days of the menstrual cycle (in this review denoted as the early follicular phase) are characterized by low serum levels of estradiol, around or below 200 pmol/l, but during the first days of menses it is not uncommon to encounter estradiol serum concentrations in the postmenopausal range, i.e., below 100 pmol/l (**Figure 1**). With the rise of a dominant follicle, estradiol levels rapidly increase during the second week of the menstrual cycle (late follicular phase), and during the pre-ovulatory estradiol surge levels between 600 and 2500 pmol/l, or even higher, may be encountered (Schuster et al., 2010). The estradiol peak is followed 12–24 h later by the luteinizing hormone (LH) surge, and ovulation, in turn, occurs typically 10–12 h after the LH surge (Speroff and Fritz, 2010). The LH surge can be measured by urinary LH kits, and it is generally advised to start daily tests already on menstrual cycle day 10, in order to capture the LH surge also in women with slightly shorter cycles. In the clinic, a positive LH surge is sufficient for diagnosis of an ovulatory cycle, although it is sometimes argued that the final proof of ovulation is the progesterone secretion of the luteal phase (Speroff and Fritz, 2010).

Following ovulation, the dominant follicle develops into a corpus luteum which is capable of estradiol as well as progesterone synthesis (Speroff and Fritz, 2010). Progesterone, at this stage, is needed for endometrial preparation for implantation, in case of conception, and the progesterone peak on menstrual cycle day 21 (or LH +8) coincides with the endometrial implantation window on menstrual cycle day 21 (LH +8) (Nikas and Makrigiannakis, 2003). If LH kits are not used, ovulation can be confirmed by measurement of progesterone. In the clinic, a progesterone serum concentration above 25 nmol/l on cycle day 21 is proof of an ovulatory cycle (Speroff and Fritz, 2010), but progesterone levels >10 nmol/l (taken at some point during the luteal phase) together with a report on normal cycle length can also be used as an indicative of an ovulatory cycle (Nevatte et al., 2013). Although estradiol generally has attracted more scientific attention than progesterone in menstrual cycle studies, it should be noted that the mid-luteal progesterone serum concentration is approximately 100-fold greater than the estradiol levels at the same time-point.

Great inter-individual differences in menstrual cycle length and hormone levels are, however, at hand. In young women, deviations from the typical 28-day menstrual cycle is due to a shorter or prolonged follicular phase (Lenton et al., 1984a), whereas the

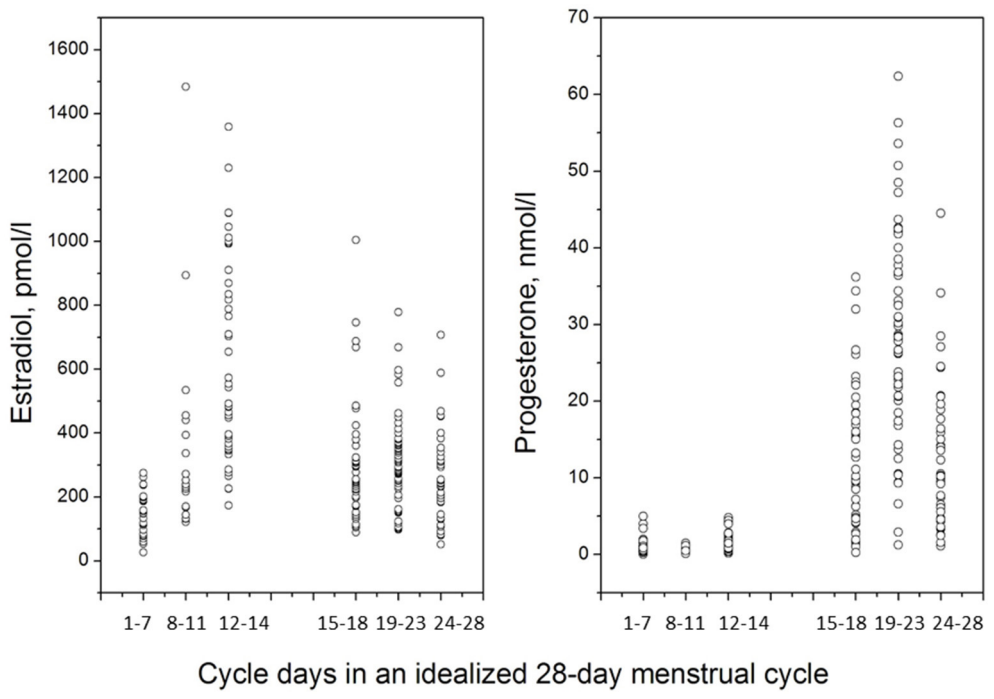

FIGURE 1 | Estradiol and progesterone levels across the menstrual cycle, frequently sampled in 47 healthy women, 18–42 years old, with self-reported history of regular menstrual cycles, and no hormonal use. Cycle phase staging was accomplished by forward counting from onset of menstrual cycle (days 1–7), backward counting from day of LH surge (days 8–11, and days 12–14), forward counting from LH surge (days 15–18, and days 19–23) and backward counting from onset of next menses (days 24–28).

luteal phase is considered relatively stable with 14 days from the LH surge to onset of menses. In women approaching their forties, shorter menstrual cycle intervals may, however, also be due to a shorter luteal phase as a first sign of ovarian aging, i.e., corpus luteum insufficiency. Short luteal phases occur in approximately 5% of menstrual cycles (Lenton et al., 1984b). Also, great inter-individual variability in hormone levels are typically encountered during the peak hormone phases, and skewed distributions of estradiol and progesterone serum concentrations are typically found, **Figure 1**.

Estradiol and progesterone are both highly lipophilic and easily pass through the blood-brain barrier. In fact, animal studies and post-mortem studies in reproductive and postmenopausal women indicate that estradiol and progesterone are accumulated in the brain (Bixo et al., 1986, 1995, 1997), with the highest concentration of progesterone found in the amygdala (Bixo et al., 1997). The estradiol receptors (ERα and ERβ) and the progesterone receptors (PRA and PRB) are highly expressed in brain areas associated with reproduction, cognitive function, and emotional processing such as the hypothalamus and the limbic system (for review, see Gruber et al., 2002; Brinton et al., 2008). For example, the expression of the estradiol receptors has been demonstrated in the human amygdala, hippocampus, claustrum, hypothalamus, and the cerebral cortex. Within the human cerebral cortex, the most distinct expression of estradiol receptors is found in the temporal cortex (Osterlund et al., 2000a,b). While human studies are not available for progesterone receptors, animal data suggest that progesterone receptors are also distributed throughout the amygdala, hippocampus, hypothalamus, thalamus, and the frontal cortex (Kato et al., 1994; Guerra-Araiza et al., 2000, 2002, 2003). Additional membrane-bound receptors have emerged as potential mediators of rapid non-genomic effects of estradiol and progesterone in rodent brain, namely G protein-coupled estrogen receptors (GPERs) which are responsive to estradiol in the hippocampus, hypothalamus, cortex and substantia nigra (Hazell et al., 2009). Progesterone, on the other hand, has been reported to bind to the progesterone receptor membrane component 1 (PGRMC1) in the cerebellum, cortical regions, hippocampus, and hypothalamic nuclei (Intlekofer and Petersen, 2011). In addition, progesterone can also be metabolized into neuroactive steroids, among which allopregnanolone and pregnanolone are the two neurosteroids most studied. Neurosteroids potentiate the $GABA_A$ receptor, where they increase hyperpolarization and act in a similar manner to barbiturates and benzodiazepines (Melcangi et al., 2011). As GABA is the major inhibitory transmitter in the central nervous system, acute administration of allopregnanolone has sedative, anxiolytic, anti-convulsant properties but may also negatively influence cognitive function (Johansson et al., 2002; Kask et al., 2008a; Melcangi et al., 2011). A functionally relevant amount of allopregnanolone is synthesized in the brain, but the main source of brain and serum allopregnanolone in non-pregnant women is progesterone synthesized by the corpus luteum (Ottander et al., 2005).

METHODS

Over the past years menstrual cycle studies have greatly improved in quality, and strategies and methods for menstrual cycle studies have been established (Becker et al., 2005). Such methods include correct classification of menstrual cycle stage by use of hormonal measures (serum or saliva hormone concentrations, basal body temperature (BBT), or assessments of the LH surge) in addition to calendar-based assessments (Becker et al., 2005). Peripheral concentrations of estradiol and progesterone vary substantially between individuals (**Figure 1**), why a single measurement alone is insufficient for cycle phase determination. Instead, a combination of calendar method and cycle phase determination is needed. For follicular phase assessments, forward counting from menstrual cycle onset together with a hormonal measurement is sufficient. In the luteal phase, two different approaches are possible: (1) forward counting of days from onset of the LH surge (or BBT rise) or (2) backward counting from onset of next menses together with a progesterone assay. If hormone measures suggest that the cycle phase is incorrect subjects should, of course, be excluded.

In this study we have only accepted studies that have employed some type of hormonal assessment for confirmation of cycle phase according to previous guidelines (Becker et al., 2005). The grand majority of studies included employed either saliva or serum concentrations of hormones, but two studies relied on LH detection only (Epting and Overman, 1998; Pletzer et al., 2011) or basal body temperature (Solis-Ortiz et al., 2004; Solis-Ortiz and Corsi-Cabrera, 2008), respectively. In the manuscripts reviewed, a minority clearly stated that hormonal measurements had been used to exclude subjects who fell out-side of the stipulated cycle phases. For this reason we had to accept all manuscripts that contained some hormonal measurement, although it is unclear if these measures in all cases were acted upon.

The menstrual cycle days reported in each individual study was recalculated according to the idealized 28-day menstrual cycle to facilitate comparison of menstrual cycle stage between studies. Furthermore in this review, early follicular phase is defined as cycle day 1–7, late follicular phase as cycle day 8–14, early luteal phase as cycle day 15–21 and late luteal phase as cycle day 22–28. We have also consistently used the reproductive vocabulary, i.e., early follicular, instead of menstrual, phase.

Results from counterbalanced, longitudinal designs and cross-sectional studies have been separated, with greater emphasis on findings gained by longitudinal studies. Longitudinal studies typically find less pronounced menstrual cycle changes than cross-sectional designs, but are susceptible to training or learning effects (which may be circumvented by counterbalancing the order of study entries). Two of the longitudinal studies included were unbalanced (Becker et al., 1982; Courvoisier et al., 2013), but this has been specifically noted in the results.

The cross-sectional design, on the other hand, is susceptible to selection bias and may end up measuring effects that are more related to inter-individual performance differences than hormonal effects. For this reason the cross-sectional design is associated with an increased risk of chance findings, although this risk can be counteracted by increasing the sample size. With certain experimental set-ups it may, however, be impractical, or sometimes not even possible, to repeat experiments over time.

A meta-analysis was conducted for the error rate in mental rotation tasks by use of the Meta-analysis with Interactive

eXplanations (MIX) 2.0 Pro software package. The standardized mean difference in error rate with a 95% confidence interval (CI) was calculated on the basis of mean differences, standard deviations and the number of participants in each of the included studies.

COGNITIVE TASKS ACROSS THE MENSTRUAL CYCLE

The predominant hypotheses in the field of menstrual cycle-related cognitive changes state that: (1) Sexually dimorphic cognitive abilities/skills that favor men are improved during phases with low estrogen and progesterone levels such as the early follicular phase, (2) Sexually dimorphic cognitive abilities/skills that favor women are improved during phases with increased estrogen and/or progesterone such as the late follicular phase and mid-luteal phase. Studies along these two hypothesis has been reviewed, although not exclusively so.

VISUOSPATIAL ABILITY

Mental rotation

Men outperform women on tasks reflecting visuospatial ability, at least as long as tasks cannot be verbalized (such as object location tasks, small-scale navigation, and landmark-based navigation) (Andreano and Cahill, 2009). The most commonly used test, which also consistently differ between men and women is mental rotation (Andreano and Cahill, 2009). Findings on mental rotation performance across the menstrual cycle are summarized in **Table 1**. A mentioned concern has been that the mental rotation task should be sufficiently difficult, i.e., three-dimensional instead of two-dimensional depictions and large, as opposed to small, angular disparities should be used in order for menstrual cycle phase differences to be detected (Hausmann et al., 2000; Hampson et al., 2014). As seen in **Table 1**, because of these concerns, most researchers over the past decade have used the Shepard Metzler or Vandenberg & Kuse mental rotation tasks. While the majority of studies in **Table 1** do not demonstrate the expected improved performance in the early follicular phase, or at times of low estradiol levels (Gordon and Lee, 1993; Epting and Overman, 1998; Halari et al., 2005; Schoning et al., 2007; Mordecai et al., 2008; Kozaki and Yasukouchi, 2009; Griksiene and Ruksenas, 2011, however see Hausmann et al., 2000; Maki et al., 2002; Courvoisier et al., 2013; Hampson et al., 2014), significant methodological concerns are at hand in a significant proportion of the studies. For instance, two studies included samples sizes that were extremely small (Hausmann et al., 2000; Dietrich et al., 2001), one study used an unbalanced longitudinal design which may have opened up for training effects (Courvoisier et al., 2013), one study reported on a composite score for visuospatial tasks which may have precluded the detection of more direct effects on mental rotation (Gordon and Lee, 1993), and one study used a task originally developed for children which may have been too

Table 1 | Menstrual cycle studies on mental rotation.

Authors and design	Subjects	Cycle phases	Task	Result	Cohen's *d* (error rate)	E2 correlations[c]
LONGITUDINAL						
Gordon and Lee, 1993[a]	34 NC/34 OC	2–3/10–14/20–24	Shepard Metzler	No effect of phase or OC		
Epting and Overman, 1998	27 NC	3–4/21–22	Male figures	No effect of phase	0.06	
Hausmann et al., 2000	8 NC	2/22	Vandenberg Kuse	↑ early follicular	0.84	−0.48/−0.70*[e]
Dietrich et al., 2001	6 NC	Menses/11–12	Vandenberg Kuse	no effect of Phase		
Maki et al., 2002	16 NC	1–3/19–24	Vandenberg Kuse	↑ early follicular	0.97	−0.51*
Schoning et al., 2007	20 NC	1–3/21–25	Vandenberg Kuse	No effect of phase	0.22	
Mordecai et al., 2008	16 NC/20 OC	2–4/20–22	Vandenberg Kuse	No effect of phase or OC	0.03	
Kozaki and Yasukouchi, 2009	16 NC	1–3/high E2	Shepard Metzler	No effect of phase	0.33	
Griksiene and Ruksenas, 2011	20 NC/23 OC	2–5/14/20	Shepard Metzler	No effect of phase or OC[f]		
Courvoisier et al., 2013[b]	7 NC	Once daily 8 weeks	Shepard Metzler	↑ at low E2 phases	0.26	
CROSS-SECTIONAL						
Halari et al., 2005[c]	42 NC	3–7	Vandenberg Kuse	No hormonal correlations		−0.29
Hampson et al., 2014[c,d]	44 NC	Low-E2/highE2	Vandenberg Kuse	↑ low E2	1.14	−0.37*
			Clock rotation test, easy	No effect of phase	0.11	
			Clock rotation test, hard	↑ low E2	0.85	−0.38*

NC, normal cycling; OC, oral contraceptive users; E2, estradiol.

[a] A composite score consisting of Shepard Metzler, 3D clocks, point location, and a test of perceptual closure was reported.

[b] Unbalanced design, correlation reported for E2 and error rate.

[c] Partial correlation with control for sex hormone binding globulin (SHBG), otherwise Pearson's correlation.

[d] Low E2 had mean saliva concentration of 3.17 ± 1.0 pg/mL and high E2 6.24 ± 1.7 pg/mL, regardless if tests had been made in the follicular or luteal phases.

[e] Two correlation coefficients were reported, from the first and second test session.

[f] Third generation OC users had longer reaction times in the mental rotation task than normal cycling women, but did not differ in accuracy.

** $p < 0.05$.*

easy (Epting and Overman, 1998). In addition, the cross-sectional study by Halari included women in a very narrow time-frame in the follicular phase, whereby the possibility to detect estradiol correlations were in fact minimized (Halari et al., 2005). Finally, two of the studies were neuroimaging studies and it cannot be excluded that behavioral measures in the scanner may differ from that obtained in pure behavioral studies (Dietrich et al., 2001; Schoning et al., 2007). However, even if the studies with methodological concerns are disregarded, four out of the six remaining studies were unable to detect any menstrual cycle differences in mental rotation performance (Maki et al., 2002; Schoning et al., 2007; Mordecai et al., 2008; Kozaki and Yasukouchi, 2009; Griksiene and Ruksenas, 2011; Hampson et al., 2014).

Six of the mental rotation studies provided sufficient information for inclusion in a meta-analytic approach (Epting and Overman, 1998; Hausmann et al., 2000; Maki et al., 2002; Schoning et al., 2007; Mordecai et al., 2008; Kozaki and Yasukouchi, 2009). The meta-analysis yielded an standardized mean difference in error rate of 1.61 (95% CI −0.35 to 3.57, ns), *at present* suggesting no favor of an early follicular phase improvement in mental rotation performance. If the meta-analysis is narrowed down to studies using the Shepard Metzler or Vandenberg & Kuse tasks, this finding remains, standardized mean difference 1.73 (95% CI −0.29 to 3.76, ns). However, it should be noted that while the meta-analysis failed to provide a significant finding, this may very well be due to low power. Another finding that may suggest that further studies in this field should be pursued was that most correlational analyses revealed a negative correlation between mental rotation accuracy and estradiol levels, **Table 1**.

The neural correlates of mental rotation has been evaluated in relation to the menstrual cycle by two studies (Dietrich et al., 2001; Schoning et al., 2007). Both studies report changes in brain reactivity across the menstrual cycle and an increased reactivity in Brodmann area (BA) 39, or the angular gyrus, during presence of high levels of estradiol. Angular gyrus is involved not only in verbal processing but also in spatial judgment (Chen et al., 2012; Seghier, 2013) and according to the authors, the increased reactivity may reflect an increased need to recruit this area to solve the task at hand during the luteal phase (Schoning et al., 2007; Dietrich et al., 2001).

Yet at the same time, while the hypothesis that mental rotation performance should be superior during phases of low estrogen and progesterone levels could not be substantiated at this stage, future studies may very well alter the picture. There are several reasons for this, first it should be noted that studies that have claimed positive findings almost consistently have reported findings in the same direction, i.e., toward improved mental rotation performance in the early follicular phase. Clearly, adequately powered studies should settle this issue, and by including sufficient information on outcomes, future meta-analyses could, in fact, alter the results of the present review. Secondly, the hypothesis for mental rotation performance may have been too loosely defined. Most studies have used the mid-luteal phase as contrast to the early follicular phase, but at this stage it may be that estradiol levels are not sufficiently elevated for an effect to be noted, or that the mid-luteal progesterone surge counteracts the effect of estradiol. Finally, another interesting aspect is that women with polycystic ovary syndrome, which is characterized by hyperandrogenism (i.e., elevated androgen levels) display superior mental rotation performance in comparison with healthy, naturally cycling women (Barry et al., 2013). Maybe a more male-like performance should be expected not only in the early follicular phase but also among anovulatory women with PCOS, further emphasizing the need to evaluate not only estradiol but also testosterone.

Other visuospatial tasks

Findings on a whole range of other tasks evaluating visuospatial ability is presented in **Table 2**. Besides mental rotation, spatial tests can also broadly be categorized into tasks that evaluate spatial perception and spatial visualization, and among the latter navigation tests and object location test are included (Linn and Petersen, 1985). Notably, while men outperform women on most tasks reflecting visuospatial ability, a female advantage has been noted for tasks that can be verbalized (such as object location) (Andreano and Cahill, 2009). For this reason the hypothesis that visuospatial task performance should be superior in the early follicular phase should not include studies using the object location task, where the opposite hypothesis may be more relevant.

However, except for two studies from the same group reporting improved performance on visuospatial performance during the early follicular phase, or at times of low estradiol levels (Hampson, 1990; Hampson et al., 2014), the majority of studies have not been able to discern any menstrual cycle influence on tests of visuospatial memory or ability (Phillips and Sherwin, 1992; Gordon and Lee, 1993; Epting and Overman, 1998; Hausmann et al., 2000; Halari et al., 2005; Mordecai et al., 2008; Solis-Ortiz and Corsi-Cabrera, 2008; Weis et al., 2011), **Table 2**. Again, a number of methodological concerns have been identified in the studies; several studies suffer from low power (Hausmann et al., 2000; Solis-Ortiz and Corsi-Cabrera, 2008), two studies report on a visuospatial composite score (Hampson, 1990; Gordon and Lee, 1993), and one study involved repeated, daily testings during two menstrual cycles opening up for practice effects (Becker et al., 1982). Furthermore, because of the variety of tasks reflecting various measure of spatial perception and spatial visualization, no attempt for meta-analysis was made. Among the studies that reported a menstrual cycle influence, Hampson (1990) found improved visuospatial ability in the menstrual phase using a composite score of three different tasks (Hampson, 1990). Similarly, Hampson et al. (2014) evaluated visuospatial abilities, including mental rotation, in women with low (approximately corresponding to early follicular phase levels) and high (approximately corresponding to late follicular levels) estradiol levels, regardless if subjects had been assessed in the follicular or luteal phases of the menstrual cycle (Hampson et al., 2014). While this may be a biologically sound approach, it also serves as an example that perhaps no predictive menstrual cycle-related effects in visuospatial abilities exist, as low estradiol levels can be found during the early follicular phase, post-ovulation, the late luteal phase and during anovulatory cycles.

It may also be argued that certain math tests involve components of visuospatial ability. Two studies have evaluated such tests across the menstrual cycle (Becker et al., 1982; Pletzer et al.,

Table 2 | Menstrual cycle studies and other visuospatial tests.

Authors and design	Subjects	Cycle phases	Task	Result	Cohen's *d*
LONGITUDINAL					
Becker et al., 1982[a]	14 NC	Once daily 2 cycles	Spatial math test	↑ early follicular	
Hampson, 1990	50 NC	3–5/–16	Space relations	↑early follicular[c]	
			Rod and Frame		
			Hidden Figures Test		
Phillips and Sherwin, 1992	25 NC	3–4/19–24	Visual reproduction (VMS)	↑ early follicular	
Gordon and Lee, 1993[b]	34 NC/34 OC	2–3/10–14/20–24	Point Location	No effect of phase or OC	
			Perceptual closure	No effect of phase	
Epting and Overman, 1998	27 NC	3–4/21–22	Rod and Frame	No effect of phase	0.18
			Object location (Silverman)	No effect of phase	0.35
			Water Level	No effect of phase	0.09
Mordecai et al., 2008	16 NC/20 OC	2–4/20–22	Brief Visuospatial Memory Test	No effect of phase or OC	0.13
Solis-Ortiz and Corsi-Cabrera, 2008	9 NC	1–2/13–14/20–21/24–25	Hidden Figures Test	No effect of phase	
			Localization Test	↓ early follicular	
Weis et al., 2011	14 NC	1–3/9–11/21–23	Figure comparison test	No effect of phase	
Hausmann et al., 2000	8 NC	2/22	Hidden Figures Test	No effect of phase	0.13
CROSS-SECTIONAL					
Halari et al., 2005	42 NC	3–7	Benton line orientation	No hormonal correlations	
Hampson et al., 2014[d]	44 NC	Low E2/high E2	Perceptual closure	↑ low estradiol	

NC, normal cycling; OC, oral contraceptive users; E2, estradiol; VMS, Wechsler memory scale.

[a] Unbalanced design.

[b] Composite score consisting of Shepard Metzler, 3D clocks, point location, and a test of perceptual closure was reported.

[c] Composite score of the three tests were used for statistical analyses, a polynomal correlation with E2 was also reported.

[d] Subjects were grouped according to saliva estradiol concentrations, regardless if tests had been made in the follicular or luteal phase.

2011). Although both studies reported on superior performance in the follicular phase compared to the luteal phase, the study by Becker and colleagues had an unbalanced longitudinal design, and Pletzer and colleagues did not distinguish between the early and late follicular phase, hence the role of low estradiol was not entirely captured (Becker et al., 1982; Pletzer et al., 2011). Performance on simple mathematical calculations such a subtraction and multiplication appear not to differ across cycle phases (Hampson, 1990). No studies on menstrual cycle influence on virtual and real world navigation have thus far been reported.

VERBAL TASKS

A female advantage for verbal fluency and verbal memory is well documented (Andreano and Cahill, 2009). Accordingly, the predominant hypothesis for menstrual cycle studies on verbal fluency and memory has thus been that women should perform better at these tasks during time-periods of high estradiol levels, i.e., during the late follicular phase or mid-luteal phase.

The review of menstrual cycle studies on verbal tasks is summarized in **Table 3**. Again, a number of studies have methodological flaws including low power (Rosenberg and Park, 2002; Konrad et al., 2008; Solis-Ortiz and Corsi-Cabrera, 2008), multiple test sessions in individual subjects (Rosenberg and Park, 2002; Solis-Ortiz and Corsi-Cabrera, 2008), composite scores by which specific task performance may be disguised (Gordon and Lee, 1993), or used unwisely chosen time-frames for their assessments (Halari et al., 2005). The most frequently used tasks include verbal fluency and verbal recall, but tests reflecting semantic retrieval and implicit verbal memory have also been employed. As seen in **Table 3**, relatively few studies have documented any significant findings across the menstrual cycle and no consistent pattern, according to the above hypotheses, emerges (Hampson, 1990; Phillips and Sherwin, 1992; Gordon and Lee, 1993; Maki et al., 2002; Rosenberg and Park, 2002; Halari et al., 2005; Konrad et al., 2008; Mordecai et al., 2008; Solis-Ortiz and Corsi-Cabrera, 2008; Hatta and Nagaya, 2009; Griksiene and Ruksenas, 2011; Jacobs and D'Esposito, 2011; Hampson et al., 2014). Two imaging studies have evaluated verbal tasks across the menstrual cycle. In line with the behavioral results, Rumberg and colleagues found no difference in brain activation during verb generation between women examined in the early follicular and late follicular/early luteal phase (Rumberg et al., 2010). Dietrich, using a similar task, on the other hand reported on increased activation of a number of language related areas (BA 45/46, 6, and 40) during the late follicular phase in comparison with the early follicular phase (Dietrich et al., 2001).

However, two important points should be stressed regarding verbal skills across the menstrual cycle. First, two studies have evaluated verbal working memory, which also taps into prefrontal dopaminergic function. The Rosenberg study, albeit small in sample size, is one of few studies which demonstrated improved performance during phases of the menstrual cycle that are characterized by high estrogen levels (Rosenberg and Park, 2002). In addition, while the overall cycle effect was negative, Jacobs and D'Esposito, in fact, demonstrated that verbal working memory task performance was modulated by an interaction between

Table 3 | Menstrual cycle studies on verbal skills and verbal memory.

Authors and design	Subjects	Cycle phases	Task	Result	Cohen's *d*
LONGITUDINAL					
Gordon and Lee, 1993[a]	34 NC/34 OC	2–3/10–14/20–24	Verbosequential score	No effect of phase or OC	0.05
Griksiene and Ruksenas, 2011	20 NC/23 OC	2–5/14/20	Verbal fluency	No effect of phase, NC > OC	
Hampson, 1990	50 NC	3–5/–16	Verbal fluency	No effect of phase	
Hatta and Nagaya, 2009	30 NC	2–3/21–22	Verbal recall	No effect of phase	0.23
Jacobs and D'Esposito, 2011[b]	24 NC	1–2/11–12	Verbal working memory	No effect of phase	
Konrad et al., 2008	12 NC	1–3/19–23	Synonym generation	No effect of phase	0.04–0.23
Maki et al., 2002	16 NC	1–3/19–24	Verbal fluency	↑ midluteal	0.56
			Implicit verbal memory	↑ midluteal	0.72
			Explicit verbal memory	No effect of phase	−0.45
Mordecai et al., 2008	16 NC/20 OC	2–4/20–22	CVLT verbal recall	No effect of phase ↑ active treatment in OC	0.30
			Verbal fluency	No effect of phase or OC	0.04
Phillips and Sherwin, 1992	25 NC	3–4/19–24	Immediate paragraph recall	No effect of phase	
			Delayed paragraph recall	No effect of Phase	
			Associate verbal recall	No effect of Phase	
			Digit span	No effect of phase	
Solis-Ortiz and Corsi-Cabrera, 2008	9 NC	1–2/13–14/20–21/24–25	Verbal fluency	↑ late follicular vs. late luteal	
Rosenberg and Park, 2002	8 NC/10 OC	0, 7, 14, 21	Verbal working memory	↑ day 7 and 14 vs. day 0 and 21 No effect of OC	
CROSS-SECTIONAL					
Hampson et al., 2014[c]	44 NC	lowE2/highE2	Rhyme generation	No effect of phase	0.21
			Synonym generation	No effect of phase	−0.18
Halari et al., 2005	42 NC	3–7	Verbal fluency	No hormonal correlation	

NC, normal cycling; OC, oral contraceptive users; E2, estradiol; CVLT, California Verbal Learning Test.

[a] Composite score consisting of perception, verbal recall and verbal fluency.

[b] A COMT genotype by phase interaction was reported.

[c] Subjects were grouped according to saliva estradiol concentrations, regardless if tests had been made in the follicular or luteal phase.

COMT Val158Met and estradiol levels (Jacobs and D'Esposito, 2011). In the presence of high late follicular phase estradiol levels, an improved cognitive performance was found in Val/Val carriers (which putatively is associated with lower frontal dopamine levels), whereas a deteriorated performance was found in Met/Met carriers in comparison with the performance during the early follicular phase (Jacobs and D'Esposito, 2011). Thus, verbal working memory seems to be one verbal task were estradiol load is important, however, the genetic make-up of women may also influence the outcome. Further discussion of tasks that probe the prefrontal lobe are found below.

Secondly, although the menstrual cycle studies on verbal memory were mostly negative it should be noted that this does not rule out the possibility of an estradiol influence. Verbal memory is a relatively common cognitive outcome in randomized clinical trials on estrogen treatment in postmenopausal women, and possibly the only cognitive task where there is some evidence that estrogen treatment may have a beneficial effect. According to a recent review, there is some evidence that estrogen treatment protects verbal memory in surgically postmenopausal women (Sherwin, 2012), whereas it has no effect when initiated more than a decade after the menopause. Possibly the hormonal changes across the menstrual cycle are too swift to detect an impaired performance in the relatively short early follicular phase, especially since extremely low estradiol levels (in the postmenopausal range) not are seen in all women, and if seen, only for a few days. Evidence for this assumption may be drawn from a study on young women treated with gonadotropin releasing hormone agonists, which resulted in suppressed estradiol levels. Following eight weeks of treatment, the estradiol suppression obtained was associated with impaired verbal memory performance (Craig et al., 2007).

COGNITIVE CONTROL

Given the suggested modulatory role for estrogen (and progesterone) in the frontal dopaminergic system (reviewed in Becker and Hu, 2008), studies probing cognitive control across the menstrual cycle have also been reviewed. Cognitive control has been evaluated by use of the Wisconsin Card Sorting Test and various inhibitory tasks. While Solis-Ortiz and co-workers initially reported on improved performance in the Wisconsin Card Sorting Test during the early follicular and early luteal phase (Solis-Ortiz et al., 2004), they later failed to replicate this finding (Solis-Ortiz and Corsi-Cabrera, 2008). Using tasks that test the

ability to inhibit prepotent responses, Colzato et al. reported on less efficient inhibition in the late follicular phase (Colzato et al., 2010), whereas Bannbers and colleagues found no effect of menstrual cycle, either in accuracy or reaction time to a Go-NoGo task (Bannbers et al., 2012). However, using a task that probed inhibitory *input* control, as opposed to the Stop-signal task and Go-NoGo concerned with inhibitory *output* control, Colzato and colleagues demonstrated superior inhibition of return in the late follicular phase (Colzato et al., 2012). Also, the tendency to choose an immediate reward over greater, delayed rewards reportedly decrease between the early and late follicular phase, and this decrease is influenced by estradiol levels (Smith et al., 2014). Finally, working memory is also a prefrontal cortex-dependent cognitive function that supports an array of essential human behaviors. As already mentioned in the section on verbal tasks, verbal working memory performance appear superior at times of high estradiol levels (Rosenberg and Park, 2002; Jacobs and D'Esposito, 2011), and this may also be true for spatial working memory (Hampson and Morley, 2013).

EMOTIONAL ASPECTS OF THE MENSTRUAL CYCLE

Over the past years, the menstrual cycle influence on emotional processing, emotional memory, and fear conditioning has gained increasing interest. While findings thus far are scarce, this line of research taps into the emotional disorders of the menstrual cycle.

Many women suffer from emotional problems during the menstrual cycle. Approximately 2–10% of women in childbearing ages are afflicted by severe premenstrual symptoms, and 2–5% fulfill criteria for premenstrual dysphoric disorder (PMDD) (O'Brien et al., 2011). Population-based epidemiological studies furthermore suggest that sub-threshold PMDD may be even more prevalent, found in 18% of women (Wittchen et al., 2002). PMDD is typified by socially disrupting symptoms such as depressed mood, anxiety, and irritability which consistently appear in the late luteal phase of the menstrual cycle and remit 1–2 days after onset of menses (O'Brien et al., 2011). Premenstrual emotional disturbances have consistently been linked to progesterone exposure during the luteal phase, as symptom remit during GnRH agonist suppression of ovarian hormone levels, and are reinstated with progesterone add-back (reviewed in Nevatte et al., 2013). However, the hormone fluctuations of the menstrual cycle may also be of importance for major depressive disorder and a number of anxiety disorders, as the female bias for developing these disorders appear at menarche (suggesting activational effects of ovarian steroids) (Kessler et al., 1993), but also because many women with these disorders complain of a premenstrual worsening (Sigmon et al., 2000; Kornstein et al., 2005; Nillni et al., 2012). Overall, besides the mate preference studies (which is beyond the scope of this review, see instead Gangestad and Thornhill, 2008; Jones et al., 2008; Little, 2013), the emotional processing aspects of the menstrual cycle are far less researched than the cognitive aspects, **Table 4**. Notably, most efforts in this area has had a cross-sectional approach, and only two longitudinal studies have been identified (Conway et al., 2007; Bayer et al., 2014).

A number of studies have investigated how facial emotion recognition is affected by the menstrual cycle, **Table 4**. Compared to the midluteal phase, a better emotion recognition accuracy has been suggested in the early follicular phase (Derntl et al., 2013) and late follicular phase (Derntl et al., 2008a,b) independent of emotion stimuli, or specifically for sad faces (Guapo et al., 2009). However, when working memory for facial emotion recognition was tested, worse performance for sad and disgusted faces was detected in the early follicular phase (Gasbarri et al., 2008), possibly because the test incorporated cognitive aspects also influenced by estrogen. Hence, emotion recognition accuracy appear poorer in the luteal phase, specifically for the negative emotional stimuli (Derntl et al., 2008b) and these findings are corroborated by a report on decreased facial recognition accuracy upon acute progesterone administration (van Wingen et al., 2007). Women also demonstrate a greater tendency to perceive fearful expressions (with averted as compared to direct gaze) as more intense if progesterone levels are high (Conway et al., 2007), and respond faster to sad and angry situations or sad faces in the mid-luteal phase (Gasbarri et al., 2008; Derntl et al., 2013), as well as to other aversive stimuli, such as snakes (Masataka and Shibasaki, 2012). However, all of these studies have been performed in healthy women and it is unclear as to what extent premenstrual disorders influence the overall results of these studies. While most studies used psychiatric interviews to exclude ongoing depressive and anxiety disorders, only one study utilized daily symptom scoring for diagnosis of PMDD, and consequently, exclusion of PMDD in the control group (Rubinow et al., 2007). Not surprisingly, women with PMDD showed impaired facial emotion recognition performance and a negative bias (neutral faces being misjudged as sad) in the luteal phase, whereas the controls did not differ across cycle phases (Rubinow et al., 2007). Besides this finding, there is plenty of evidence to suggest that PMDD is associated with altered emotional processing across the menstrual cycle (Epperson et al., 2007; Kask et al., 2008b; Bannbers et al., 2011; Gingnell et al., 2012, 2013a, 2014; Hoyer et al., 2013; Comasco et al., 2014). Clearly, these findings demonstrate that premenstrual disorders need to be accounted for in menstrual cycle research on emotion processing.

An increasing interest in menstrual cycle influence on emotional memory has also been noted, **Table 4**. Emotional memory depends on hypothalamus-pituitary-adrenal (HPA) axis hormones and sympathetic activity, and a sex-related difference has been suggested (Andreano and Cahill, 2009). Again, progesterone, and the luteal phase has attracted most attention, presumably as progesterone in other species is considered a stress hormone (Fajer et al., 1971; Frye, 2007; Axner, 2008), and because of the close relationship between progesterone and the HPA axis, notably most commonly studied in relation to onset of human labor (Vrachnis et al., 2012). Across the menstrual cycle, baseline cortisol levels appear unaltered (Nepomnaschy et al., 2011), whereas cortisol reactivity to stress seem elevated in the luteal phase (Kirschbaum et al., 1999).

Results on emotional memory throughout the menstrual cycle relatively consistent. Whereas the only longitudinal study in the field recently reported decreased recognition for negative items in the luteal phase (Bayer et al., 2014), cross-sectional studies have demonstrated no difference (Felmingham et al., 2012) or enhanced memory for emotional items (Ertman et al., 2011) in

Table 4 | Menstrual cycle studies on emotion processing.

Authors and design	Subjects	Cycle phases	Design	Task	Result
EMOTION RECOGNITION					
Derntl et al., 2013	37 NC	2–5/18–25	c.s	Facial emotion recognition	↓ Accuracy mid-luteal phase
				Affective responsiveness	↓ reaction times mid-luteal phase
Conway et al., 2007	52 NC	Low P/high P	Long	Facial emotion recognition (averted gaze)	↑ fearful faces perceived as more intense in high P
Derntl et al., 2008a	32 NC	7–13/15–27	c.s	Facial emotion recognition	↓ Overall accuracy luteal phase
				Memory of emotion recognition	No difference across cycle phases
Guapo et al., 2009	30 NC	1–5/12–14/21–23	c.s	Facial emotion recognition	↓ Accuracy mid-luteal phase for sad faces
van Wingen et al., 2007	16 NC	Day 2–7	P treatment	Facial recognition	↓ Accuracy in P-treated
Gasbarri et al., 2008	56 NC	1–2/4–13/14–32	c.s	Working memory for facial emotion recognition	↓ Accuracy for sad and disgusted faces in late follicular phase
EMOTIONAL MEMORY					
Bayer et al., 2014	22 NC	1–4/17–23	Long	Emotional memory (IAPS)	↓ Midluteal recognition for negative items
Ertman et al., 2011	60 NC	1–14/15–28	c.s.	Emotional memory (IAPS)	↑ Luteal free recall of negative items
Nielsen et al., 2013	42 NC/36 OC	1–14/15–28	c.s.	Emotional memory (narrative)	↑ Luteal memory for peripheral details
Andreano et al., 2008	64 NC	1–7/8–13/18–24	c.s.	Emotional memory (narrative) ± CPS	No effect of stress or cycle phase on recall
Felmingham et al., 2012	56 NC	High P/low P	c.s.	Emotional memory (IAPS) ± CPS	↑ memory during stress in high P
Kuhlmann and Wolf, 2005	27 NC/20 OC	2–4/20–24	Cortisol/placebo	Emotional memory (negative/neutral words)	No difference in cortiol-induced retrieval impairment
FEAR LEARNING					
Merz et al., 2012	60 NC/30 OC	3–8/20–26	c.s	Fear conditioning + cortisol/placebo	No difference across cycle phases
Milad et al., 2010	36 NC	High E2/low E2	c.s	Fear conditioning + fear extinction	No difference between groups
Zeidan et al., 2011	34 NC	High E2/low E2	c.s	Fear conditioning + fear extinction	No difference between groups
				Fear extinction recall	↓ recovery of fear in high E2
Graham and Milad, 2013	31 NC	Day 1–5	E2 treatment	Fear conditioning + fear extinction	No difference
				Fear extinction recall	↓ recovery of fear in E2-treated
SPONTANEOUS INTRUSIVE RECOLLECTIONS					
Ferree et al., 2011	54 NC	1–13/15–28	c.s	Film clips	↑ SIR in early luteal phase
Soni et al., 2013	41 NC	7–11/16–20/24–28	c.s	Film clips	↑ SIR in early luteal phase

NC, normal cycling; OC, oral contraceptive users; E2, estradiol; P, progesterone, c.s., cross-sectional; long, longitudinal-balanced; SIR, Spontaneous intrusive recollections;, IAPS, International Affective Picture System; CPS, cold pressor stress.

the luteal phase. Furthermore, enhanced memory for peripheral details of an emotional story has been linked to the luteal phase (Nielsen et al., 2013) and emotional memory correlated positively with progesterone levels sampled at the time of encoding (Ertman et al., 2011).

Spontaneous intrusive recollections (SIR) are known to follow emotional events in clinical and non-clinical populations, among the former, posttraumatic stress disorder is the most obvious example. Indeed, also the menstrual cycle may influence such recollections in trauma patients (Bryant et al., 2011) and as well as in healthy women (Ferree et al., 2011; Soni et al., 2013). These flashbacks or spontaneous intrusive recollections appear to be more common if the trauma or experimental exposure to aversive stimuli occurred in the luteal phase (Bryant et al., 2011; Ferree et al., 2011; Soni et al., 2013), and again, progesterone levels were positively correlated with SIR frequency (Ferree et al., 2011).

In addition, various strategies to modulate the HPA axis across the menstrual cycle have been employed, for instance by cortisol treatment or by use of stress tests such as the Cold Pressor Stress test (CPS). Kuhlmann and colleagues reported that cortisol

treatment resulted in impairment of emotional verbal memory, but found no difference in the cortisol-induced memory impairment between women assessed in the follicular or luteal phases (Kuhlmann and Wolf, 2005). While one study reported that women with high progesterone levels had greater memory recall for negative images if subjected to post-training CPS physical stress (Felmingham et al., 2012), others found no difference across cycle phases in memory performance following CPS (Andreano et al., 2008). Andreano and colleagues, however, demonstrated that post-CPS cortisol was correlated with memory retrieval in the luteal phase although not in the follicular phase (Andreano et al., 2008).

Fear conditioning is often used as a model for the formation of emotional memories (LeDoux, 2000). By using a 2-day fear conditioning paradigm consisting of fear conditioning, and extinction learning on the first day, and extinction recall and fear renewal on the second day, Milad and others have over time presented relatively consistent results as to the estradiol involvement in consolidation or maintenance of extinction, whereas fear acquisition and fear extinction appear not to be influenced by hormonal state (Milad et al., 2010; Zeidan et al., 2011; Merz et al., 2012; Graham and Milad, 2013), **Table 4**. Their studies have suggested greater extinction memory (i.e., less recovery of fear upon fear renewal) in women during the late follicular phase or in women with high estradiol levels (irrespectively of cycle phase) (Milad et al., 2010; Zeidan et al., 2011; Graham and Milad, 2013), corroborated by the finding that a single estradiol administration during the early follicular phase resulted in similarly enhanced consolidation of extinction (Graham and Milad, 2013). No effect of progesterone or the luteal phase was noted (Milad et al., 2010; Zeidan et al., 2011). Thus, maintenance of fear extinction which is an important goal of cognitive behavioral therapy appears superior if applied in the follicular rather than in the luteal phase.

Clearly, all of these findings point toward altered emotion processing across the menstrual cycle, which is in line with the clinically relevant emotional disturbances that are reported by a substantial fraction of women. Further studies in this area could help explain the underlying mechanisms of emotional problems in the luteal phase. However, longitudinal studies are awaited, and preferably authors should investigate to which extent also PMDD (or sub-threshold PMDD) influence their results. This could be relatively easily achieved, for instance by asking women to keep daily prospective records of mood symptoms throughout the menstrual cycle in which they are tested.

The luteal phase is dominated by elevated progesterone and estradiol, and without hormonal interventions, it may be difficult to disentangle which of these two hormones is driving the results. For instance, while it is generally accepted that progesterone is the symptom provoking hormone in PMDD, hormone interventions have suggested that also estradiol plays a role. Segebladh and colleagues treated PMDD women with GnRH agonists for hormone suppression and evaluated the return of symptoms when women were exposed to three different hormonal treatments. The combination of a high estrogen dose (and progesterone) was more symptom provoking than a low estrogen dose together with progesterone (Segebladh et al., 2009). Possibly, as one of the most important aspects of estrogen action (in the brain and elsewhere) is to up-regulate progesterone receptors, increased availability to estrogen may thus result in more progesterone receptors for progesterone to act upon.

Progesterone is also associated with more complex actions than estradiol. On the one hand it has been associated with anxiolytic and sedative effects, on the other hand also with anxiogenic and depressive states. For the interested reader, several reviews on these conflicting aspects of progesterone action are available (Sundstrom Poromaa et al., 2003; Backstrom et al., 2011). In addition, it should be pointed out that women with premenstrual disorders experience their most intense symptoms in the late luteal phase, when progesterone levels are declining, not at the progesterone peak (Nevatte et al., 2013).

fMRI STUDIES ON EMOTION PROCESSING DURING THE MENSTRUAL CYCLE

Neuroimaging techniques, such as functional magnetic resonance imaging (fMRI), are useful tools to gather further insight into CNS processing. The effects of the menstrual cycle and other hormone interventions during both emotional and cognitive tasks was recently reported (Toffoletto et al., 2014), and this review merely highlights studies which correspond to previously reported behavioral data, **Table 5**. Most studies have used a longitudinal approach with repeated scanning sessions in the same participants, but the use of different tasks and contrasts for generation of the fMRI results, as well as variations in time points for assessments hampers the comparability of studies. However, some tentative conclusions may be drawn.

A pattern of increased amygdala reactivity to negative emotional stimuli in the luteal phase appears in several (Andreano and Cahill, 2010; Gingnell et al., 2012; Bayer et al., 2014), but not in all (Gingnell et al., 2013a,b) studies. A sensitivity in the amygdala to increases in progesterone is also supported by the increased reactivity in the amygdala, fusiform gyrus, inferior frontal gyri, cerebellar vermis, and supplementary motor area observed after acute progesterone administration (van Wingen et al., 2008). It should also be noted that the decrease in amygdala reactivity reported by Gingnell et al. is most likely due to habituation, as this study was not counterbalanced for phase of entry (Gingnell et al., 2013b). One cross-sectional study by Derntl et al. (2008a) report the opposite pattern with amygdala reactivity to facial stimuli being higher in the follicular than in the luteal phase. Being one of the core structures in the fear network (Shin and Liberzon, 2010) the sensitivity in the amygdala to changes in ovarian steroid hormones across the menstrual cycle fits nicely with the increased susceptibility to anxiety and depressive symptoms in the luteal phase. Two studies have assessed brain reactivity during positive stimuli and do not report menstrual cycle differences in amygdala reactivity, but increases in the ACC reactivity during the luteal phase (Protopopescu et al., 2005; Amin et al., 2006). In response to negative emotional stimuli, the ACC reactivity has been reported to decrease in the late follicular phase (Goldstein et al., 2005).

Bayer et al. reported impaired recognition of negatively valenced stimuli in the mid luteal phase, but otherwise no differences in behavior have been reported in the longitudinal fMRI

Table 5 | Menstrual cycle studies including fMRI with emotional stimuli.

Author	Subjects	Cycle phases	Task	Behavioral results	Contrast	ROI analyses	Whole Brain analyses
Gingnell et al., 2013a	14 NC	6–12/22–27	Neg. and pos. images and the anticipation thereof	No effect of phase	Neg. > pos.	n.a.	None
Protopopescu et al., 2005	12 NC	8–12/–23–27	Go NoGo emotional words	n.a.	Neg. Go > neu Go	n.a.	↑↓ OFC in the luteal phase
					Pos. Go > neu Go	n.a.	↑ ACC in the luteal phase
					Neg. NoGo > neu NoGo	n.a.	↑ OFC in the luteal phase
Gingnell et al., 2012	15 NC	6–12/22–27	Facial emotion recognition	No effect of phase	Angry & afraid faces > shapes	↑ amygdala in luteal phase	n.a.
Gingnell et al., 2013b	17 NC	1–10/15–21	Facial emotion recognition	no effect of phase	Angry & afraid faces > shapes	↓ amygdala in luteal phase	n.a.
Andreano and Cahill, 2010	17 NC	1–7/18–24	Neg. and neutral images	n.a.	Neg. > neutral	↑ amygdala and hippocampus in luteal phase	↑ IFG, fusiform gyrus, cerebellum, CN in the luteal phase
Goldstein et al., 2005	12 NC	2–3/16–18	Neg. and neutral images	n.a.	Neg. > neutral	↑ BA 40, BA 423 in late follicular ↓ ACC, hypothalamus, brain stem, OFC, amygdala, BA 47, BA 10, BA 18, BA 19, BA 37, BA 30, BA 23, BA 39, cerebellum in late follicular	n.a.
Bayer et al., 2014	22 NC	0–4/17–23	Encoding of neg., pos. and neutral images	↓ recollection of negative stimuli in luteal phase	Emotional (hit>miss) > neutral (hit>miss)	↓ hippocampus in luteal phase	n.a.
					Negative (hit>miss) > neutral (hit>miss)	↑ amygdala in luteal phase	n.a.
					Positive (hit>miss) > neutral (hit>miss)	↓ ACC in the luteal phase	n.a.
Rupp et al., 2009	10 NC	10–12/19–23	Evaluation of attractiveness in houses and faces	No effect of phase	Faces > houses	n.a.	↓ OFC in luteal phase
CROSS-SECTIONAL							
Derntl et al., 2008a	22 NC	1–14/15–28	Facial emotion recognition	↓ recognition accuracy in luteal phase	Emotional faces > cross hair	↓ amygdala in the luteal phase	↓ hippocampus in the luteal phase
					Disgusted faces > cross hair	n.a.	↓ fusiform gyrus in the luteal phase
					Sad faces > cross hair	n.a.	↓ MTG in the luteal phase
					Neutral faces > cross hair		↓ hippocampus in the luteal phase
Zeidan et al., 2011	34 NC	Low E2/High E2	Fear conditioning and extinction	No effect of group	CS+ > CS-	None	n.a.
				No effect of group	Late CS+E > early CS+E	↑ mPFC in high E2	n.a.
				↑ extinction memory in high E2	first CS+E >first CS+NE	↑ mPFC and amygdala in high E2	n.a.

NC, normal cycling; E2, estradiol; neg., negative; pos., positive; neu, neutral; ACC, anterior cingulate cortex; dmPFC, dorsomedial prefrontal cortex; dlPFC, dorsolateral prefrontal.

studies evaluating menstrual cycle effects (Bayer et al., 2014). One reason for this may of course be lack of power. Due to costly and time-consuming procedures, imaging studies tend to include few participants. It may also be that brain reactivity represents a more sensitive measure of the ovarian steroid hormone influence. This is in parallel to the increased emotion-induced amygdala reactivity found in non-depressed, non-anxious healthy carriers of the short version of the serotonin transporter promoter length polymorphism (Hariri et al., 2002; Fakra et al., 2009). However, the lack of differences in behavioral measures may also be due to the fact that healthy women are capable of using compensatory mechanisms to adjust for the different hormonal exposures. This further highlights the importance of the chosen paradigms for fMRI and that due care should be made to assure that the used stimuli is relevant to the mechanism that is to be studied.

In conclusion, the hitherto performed fMRI-studies rarely report of behavioral differences across phases, but indicate that brain reactivity may differ. The most consistent finding so far appears to be an increased amygdala response to negative emotional stimuli in the luteal phase, and an increase in ACC reactivity during the processing of positive stimuli in the luteal phase, but the lack of replications, and differences in used paradigms limits the conclusions that can be drawn.

CONCLUSION

The menstrual cycle remains an intriguing, natural experiment of relevance to many researchers in medical and psychological disciplines. While the earliest reports on menstrual cycle findings were devoted to explore the suitability of women in work-life and areas dominated by males (Sommer, 1973), the past years research appear driven by the increasing interest in sex influences on neurobiology. However, despite its' immediate appeal and accessibility, menstrual cycle studies require skillful and meticulous handling (Becker et al., 2005), and positive findings have in many cases turned out to be notoriously difficult to replicate.

According to this review, the best evidence suggest that differences across the menstrual cycle in sexually dimorphic tasks, such as mental rotation, visuospatial ability, verbal memory and verbal fluency, are small and difficult to replicate. This finding is partly in line with previous reviews which have either suggested no influence of the menstrual cycle (Sommer, 1973), or some influence although at the same time emphasizing that such changes would not be clinically relevant (Sherwin, 2012). Also, in perspective of the difficulties in establishing a firm role for estrogen treatment on cognitive function in postmenopausal women (Sherwin, 2012) (some evidence suggest a positive influence by estrogen on verbal memory if estrogen treatment is initiated in close temporal relationship with the menopause, Sherwin, 2012), it may not come as a surprise that menstrual cycle studies have, for the most part, failed to prove a menstrual cycle influence on cognitive function. Factors contributing to this may include the younger age of women included in menstrual cycle studies (assuming that a positive or protective effect of estrogen would be more difficult to detect in young women due to roof effects, i.e., a majority of women investigated at an age when cognitive decline has not yet set in), or that longer exposure of estrogen (or estrogen deficiency) is needed for detection of any cognitive effects, or that the grand majority of menstrual cycle studies have been underpowered to detect the presumably relatively small or modest effect sizes across cycle phases. Another reason why findings in menstrual cycle studies probing cognitive function have been difficult to replicate may also be the genetic make-up of women. Recently, Jacobs and D'Esposito demonstrated that prefrontal cortex activation in relation to a working memory task was modulated by an interaction between *COMT* Val158Met and estradiol levels (Jacobs and D'Esposito, 2011). Similarly, the tendency to choose an immediate reward over greater, delayed rewards decreases between the early and late follicular phase, and this decrease was influenced by estradiol levels and driven by Val/Val carriers of the *COMT* Val158 Met genotype (Smith et al., 2014). Finally, in line with the above findings, yet another reason for inconsistent menstrual cycle effects could be that estradiol and/or progesterone act as modulators for other, classical neurotransmitters. For instance, in the above cited studies, the effect of estradiol putatively depends on frontal dopamine levels. Given the wide-spread interactions of estradiol and progesterone on the serotonin neurotransmitter system (Bethea et al., 2002), GABA (Sundstrom Poromaa et al., 2003), and neuropeptides such as brain-derived neurotrophic factor (Comasco et al., 2014), additional complexity is brought into the picture. Disentangling these relationships may help in in understanding why behavioral effects of ovarian steroids are detected only inconsistently.

Further studies on the menstrual cycle modulation of emotional processing are also of importance, as they may ultimately be relevant to women's mental health. Premenstrual disorders, i.e., premenstrual syndrome and premenstrual dysphoric disorder are relatively common in women of fertile ages, and represent a great burden for afflicted women, often associated with impaired social or work-related functioning. This review has emphasized that the luteal phase is associated with impaired emotion recognition accuracy (Conway et al., 2007; van Wingen et al., 2007; Derntl et al., 2008b, 2013; Gasbarri et al., 2008; Guapo et al., 2009) and enhanced emotional memory. Emotional events that occur during the luteal phase more often result in spontaneous intrusive recollections (Ferree et al., 2011; Soni et al., 2013), and traumatic flashback memories are more common when the trauma takes place in the luteal phase (Bryant et al., 2011). In addition, a number of studies have pin-pointed progesterone as the driving factor for these findings; progesterone levels correlated with emotional memory (Ertman et al., 2011) and positively predicted intrusive memories (Ferree et al., 2011). A number of imaging studies have also reported on increased luteal phase reactivity in core structures of the fear network such as amygdala (Andreano and Cahill, 2010; Gingnell et al., 2012; Bayer et al., 2014) and the anterior cingulate cortex (Protopopescu et al., 2005; Amin et al., 2006). Again, progesterone appear a key factor for this finding as increased amygdala reactivity became evident following progesterone administration in the early follicular phase (van Wingen et al., 2008). Taken together, these findings suggest that progesterone, or at least the combined effect of estradiol and progesterone of the luteal phase, have the ability to influence various aspects of emotional processing, which may have repercussions for the clinical presentation of emotional disturbances in the luteal phase. Further studies in this area are awaited.

In conclusion, to the extent that behavioral changes have been demonstrated over the course of the menstrual cycle, the best evidence suggests that differences in sexually dimorphic tasks are small and difficult to replicate. However, emotion-related changes are more consistently found, and are better associated with progesterone than with estradiol, such that high progesterone levels are associated with increased amygdala reactivity and increased emotional memory.

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Progesterone mediates brain functional connectivity changes during the menstrual cycle—a pilot resting state MRI study

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The growing interest in intrinsic brain organization has sparked various innovative approaches to generating comprehensive connectivity-based maps of the human brain. Prior reports point to a sexual dimorphism of the structural and functional human connectome. However, it is uncertain whether subtle changes in sex hormones, as occur during the monthly menstrual cycle, substantially impact the functional architecture of the female brain. Here, we performed eigenvector centrality (EC) mapping in 32 longitudinal resting state fMRI scans of a single healthy subject without oral contraceptive use, across four menstrual cycles, and assessed estrogen and progesterone levels. To investigate associations between cycle-dependent hormones and brain connectivity, we performed correlation analyses between the EC maps and the respective hormone levels. On the whole brain level, we found a significant positive correlation between progesterone and EC in the bilateral dorsolateral prefrontal cortex (DLPFC) and bilateral sensorimotor cortex. In a secondary region-of-interest analysis, we detected a progesterone-modulated increase in functional connectivity of both bilateral DLPFC and bilateral sensorimotor cortex with the hippocampus. Our results suggest that the menstrual cycle substantially impacts intrinsic functional connectivity, particularly in brain areas associated with contextual memory-regulation, such as the hippocampus. These findings are the first to link the subtle hormonal fluctuations that occur during the menstrual cycle, to significant changes in regional functional connectivity in the hippocampus in a longitudinal design, given the limitation of data acquisition in a single subject. Our study demonstrates the feasibility of such a longitudinal Resting-state functional Magnetic Resonance Imaging (rs-fMRI) design and illustrates a means of creating a personalized map of the human brain by integrating potential mediators of brain states, such as menstrual cycle phase.

Keywords: menstrual cycle, RS-fMRI, functional connectivity, estradiol, progesterone

INTRODUCTION

Sex hormones influence both brain and behavior and are potent modulators of brain plasticity across the life-span (Peper et al., 2011; Galea et al., 2013). Several lines of evidence from animals and humans suggest sex hormones stimulate neurogenic cascade processes by promoting neurite outgrowth (Minano et al., 2008), mitochondrial and synaptic health (Hara et al., 2014), dendritic branching (Hao et al., 2006) and myelination (Patel et al., 2013), thus playing a pivotal role in structural brain organization. Neuroimaging studies in humans have begun to address the question of whether the effect of sex hormones on neural plasticity is reflected in structural connectivity changes. Supporting evidence stems from reports of increased hippocampal volume in postmenopausal estrogen therapy users (Eberling et al., 2003; Boccardi et al., 2006; Lord et al., 2008), as well as from studies demonstrating hormonal contraceptive use to be associated with larger gray matter volumes in cortical regions (Pletzer et al., 2010; De Bondt et al., 2013a), and with changes in cerebral white matter (De Bondt et al., 2013b).

The menstrual cycle offers a unique experimental-setup for studying the potential effects of subtle physiological fluctuations of sex hormones on structural or functional brain architecture. FDG-glucose positron emission tomography (PET) data suggests that, in primates, fluctuations of ovarian hormones across

the menstrual cycle influence activity in brain areas involved in the processing and regulation of emotion (Rilling et al., 2008). Glucose metabolism in the human brain also displays changes associated with the menstrual-cycle (Reiman et al., 1996). Reiman and colleagues found significantly higher glucose metabolism in the thalamus, as well as the prefrontal, temporo-parietal, and inferior temporal cortex during the mid-follicular phase, when estrogen levels are high. In contrast, higher metabolic rates were found in the anterior insula, as well as the superior temporal, anterior temporal, occipital, cerebellar, and cingulate cortex, during the mid-luteal phase (Reiman et al., 1996). A study using PET with high-specific-activity [^{11}C]raclopride showed sex differences in dopamine release between men and women (Munro et al., 2006). Baseline striatal dopamine binding potential and dopamine release in men and women following amphetamine and placebo challenges were compared. There was no sex difference in baseline binding potential, but men had greater dopamine release than women in the ventral striatum and additionally in three of four striatal regions examined. Differences were revealed in the anterior putamen, as well as the anterior and posterior caudate nuclei, but not in the posterior putamen (Munro et al., 2006).

In female cynomolgus monkeys an effect of menstrual cycle phase on dopamine D2 receptor availability in the caudate nucleus and putamen could be demonstrated (Czoty et al., 2009). Using the selective D2-like receptor ligand [^{18}F]fluoroclebopride PET scans were conducted during the luteal phase and during the follicular phase of the menstrual cycle. Distribution volume ratios for the caudate nucleus and putamen were calculated using the cerebellum as the reference region and were used as a measure of D2 receptor availability. [^{18}F]fluoroclebopride distribution volume ratios were significantly higher in the luteal phase compared to the follicular phase in both the caudate nucleus (11.7% difference, $p = 0.02$) and putamen (11.6% difference, $p = 0.03$) (Czoty et al., 2009). The authors conclude that menstrual cycle may influence striatal dopamine receptor availability.

Resting-state functional Magnetic Resonance Imaging (rs-fMRI) focuses on the assessment of spontaneous low frequency fluctuations in brain activity, in the absence of a task (Biswal et al., 1995). Measures of connectivity between these spontaneous fluctuations have been shown to reflect communication across large-scale networks in the human brain (Nir et al., 2008; Biswal et al., 2010; Keller et al., 2013). Further, sexual dimorphism has been described for these intrinsic connectivity patterns (Biswal et al., 2010; Tian et al., 2011). Given the wide expression of receptors for both estrogen and progesterone in the human brain, including many highly interconnected regions (McEwen, 2002; Brinton et al., 2008; Weiser et al., 2008), fluctuations of ovarian hormones related to the menstrual cycle likely influence the nature of communication between these brain regions. Intrinsic functional connectivity based on fMRI is especially sensitive to such coupling dynamics and can provide information about network relationships on a whole brain level (Buckner et al., 2013).

One common method to assess global network connectivity is to calculate eigenvector centrality (EC) (Lohmann et al., 2010; Sato et al., 2014), a graph-based measure of centrality that also takes the centrality of nodes that it connects to into account (Bonacich, 1972). In graph theory, a network is defined as a collection of items (nodes) that possesses pairway relationships (edges) (Sato et al., 2014). The EC of a node is proportional to the EC of the nodes in its neighbors and measures how the neighbors of a node are connected to the network (Bonacich, 1972). Moreover, EC quantifies the hierarchical relevance of a node. As we aim for a whole brain investigation of hierarchical network changes across the menstrual cycle, we chose not to apply other graph-based centrality methods such as degree, closeness or betweeness, which are less suitable for whole brain maps due to computational complexity (Lohmann et al., 2010). Furthermore, we refrain from using model-free approaches, such as independent component analysis (ICA), as for successful ICA-application substantial a posteriori selection of valid components must be made (reviewed by Margulies et al., 2010). Instead, EC is parameter-free, computationally fast and does not depend on prior assumptions (Lohmann et al., 2010). In a functional context, EC has been shown to be more sensitive to paralimbic and subcortical regions (brain regions particularly rich in sex hormone receptor density) (Zuo et al., 2012). Changes in EC signal have previously been linked to developmental changes of the brain (Sato et al., 2014), changes in motor function (Taubert et al., 2011) and pharmacologically induced changes in neurotransmitter levels (Schaefer et al., 2014). We acknowledge that EC is a measure that can be viewed as difficult to relate to function in a particular cognitive domain or to specific behavioral aspects. Buckner et al. (2013) propose in their expert-consensus statement that intrinsic functional connectivity provides a powerful and unique tool to provide insight into human brain organization. They further discuss this technique as based on an inherently ambiguous measure that reflects constraints both from static anatomical connectivity and from poorly understood dynamic functional coupling changes and recommend using this measure as a suitable tool for generating hypotheses about brain organization that will require further study.

To date, two recent studies have investigated resting state connectivity data across the menstrual cycle in humans (Petersen et al., 2014; Hjelmervik et al., 2014). The first (Hjelmervik et al., 2014) did not find any evidence for functional connectivity changes across the menstrual cycle. However, the authors limited the monitoring of the menstrual cycle to subjective subject-reporting, assessment of sex hormone fluctuation across the menstrual cycle to the collection of saliva samples of estrogen and progesterone at three time-points and did not include any LH measurements to further confirm ovulation. The analysis of the second study (Petersen et al., 2014) was constrained to the anterior default mode network (aDMN) and the executive control network, in a between-group design. The authors found greater connectivity between the aDMN and the angular gyrus in women scanned in the follicular phase vs. women scanned in the luteal phase (Petersen et al., 2014).

There are also EEG-measurements in resting humans (rest is typically dominated by alpha oscillations): Brotzner et al. (2014) correlated frequency of alpha oscillations in resting women with menstrual cycle phase, sex hormone level, or use of oral

contraceptives and suggest a modulation of resting alpha frequency by endogenous estradiol. There was no association with endogenous progesterone in this study. Luteal women showed highest and late follicular women showed lowest individual alpha frequency or center frequency that both correlated negatively with endogenous estradiol level, but not with endogenous progesterone (Brotzner et al., 2014).

Several important conclusions on the role of sex hormones in neuroplastic adaptation have also been drawn from studying the menstrual cycle in animal models, such as rats (Tanapat et al., 1999; Scharfman et al., 2003) or macaque monkeys (Czoty et al., 2009). However, in contrast with the human cycle, the rat cycle is much shorter, consisting of 4–5 days, and also the macaque estrus displays distinct differences compared to the human cycle that can complicate direct translation of such findings across species. Thus, there is substantial need for detailed studies *in vivo* in the human menstrual cycle. Most studies that demonstrate menstrual cycle related changes in brain structure and function report such observations in the context of another main hypothesis aimed at comparing men or women or women on and off oral contraception (Pletzer et al., 2010; De Bondt et al., 2013b; Petersen et al., 2014).

One of the reasons explaining this lack of neuroimaging data on the impact of the menstrual cycle on the human brain is likely tied to the challenges of thoroughly monitoring the menstrual cycle. An extensive body of literature from research on accuracy for reports of the last menstrual period before a pregnancy suggests that subjective menstrual diaries are highly prone to inaccuracies. For example, nearly 50% of women enrolling in a study examining routine ultrasound screening for prediction of gestational age had suspect menstrual histories (Campbell et al., 1985). Only 39% of women correctly reported their last menstrual bleeding when the time of recall was 3 weeks or longer, most women tended to underreport the length of their menstrual cycles at that time (Wegienka and Baird, 2005). Daily measurements of core body temperature have been proposed as a non-invasive method to monitor the menstrual cycle. However, this method has also been demonstrated to be prone to error and misreports of cycle-length because of the day-to-day variability of temperature readings, cycle variability and the effects of illness, medication, diet and changes in sleeping patterns (Bauman, 1981; Leader et al., 1985). The menstrual cycle can be divided into two main phases: the follicular phase between onset of menses and ovulation with rising levels of estrogen and very low levels of progesterone, and the luteal phase, starting after ovulation until the onset of the next menses characterized by high levels of progesterone and low levels of estrogen, especially premenstrual. Thus, detection of sex hormone concentrations in blood at single time-points to differentiate the follicular from the luteal phase is not a feasible solution, either, as very similar estrogen concentrations can be found during the late follicular and the mid-luteal phase and more frequent samples are needed to establish luteal-phase length and adequacy. Confirmation of luteinizing hormone (LH)-concentration peak in urine samples collected at mid-cycle (12–16 days in a typical cycle) represents a relatively non-invasive option to determine ovulation, because an LH surge is a necessary prerequisite for ovulation. To accurately stage the menstrual cycle phase for the current project we used a combination of all of these methods.

Detailed longitudinal studies of individual cases with rs-fMRI are not common, however there is evidence supporting such a longitudinal approach to be useful. One famous example derived from the field of structural imaging is the single-subject template "Colin's Brain" (Holmes et al., 1998), a widely used brain atlas based on 27 longitudinal scans of the same individual that allows for a high resolution and fine structural details to be seen.

Our current research was motivated by the lack of data on the potential impact of the menstrual cycle on functional brain architecture as a primary research question. To our knowledge no study to date has addressed the question whether menstrual cycle related changes in endogenous sex hormones impact intrinsic connectivity in the female brain using an extensive intra-individual longitudinal design on a whole brain level. The purpose of applying such a study-design is to address the following aspects: (1) The menstrual cycle offers a unique experimental set-up to study changes in endogenous sex hormones. However, these changes are expected to be subtle, thus an intra-individual longitudinal design will be beneficial to adequately capture such potential effects. (2) The menstrual-related changes are expected to occur within hours or days and recent evidence points toward feasibility to visualize changes in intrinsic connectivity following alteration in the neurochemistry of the brain on such a short-time scale (Schaefer et al., 2014). The implementation of shortly-timed intervals of scanning-sessions is a pre-requisite to assess changes on such a short time-scale. We timed our scanning intervals to occur every 2–3 days to account for this requirement.

The aim of the current study was to test the feasibility of such an intra-individual longitudinal design by calculating EC, a measure of intrinsic connectivity across the whole brain. We hypothesize the physiological fluctuations of endogenous estrogen and progesterone levels during the menstrual cycle to significantly impact intrinsic connectivity across the entire female brain.

METHODS

We repeatedly scanned a single, healthy 32-year old female subject with a documented history of regular menstrual cycles. Exclusion criteria were a history of psychiatric and neurological illness or the suffering from other chronic illnesses. The participant was screened using the Structured clinical interview for DSM to disqualify any Axis I major mental disorders (First et al., 1955a), supplemented by the exclusion of Axis II personality disorders (First et al., 1995b). Additionally, we used the Hamilton Rating Scale for Depression (HAM-D) to detect symptoms of clinical depression (Hamilton, 1960), the Mood Spectrum self-report (MOODS-SR) to exclude any subclinical manic-hypomanic symptoms (Dell'Osso et al., 2002), and the State-Trait anxiety inventory (STAI) to screen for clinical anxiety-symptoms (Spielberger and Vagg, 1984). The subject was at a normal weight (BMI = 20.2), antidepressant-naive, free of any medication including any contraceptives, and never pregnant or breast-feeding. Furthermore, the participant was a non-smoker and did not have any current or history of alcohol or any other substance abuse. The participant provided written informed consent to participate. Study and recruitment procedures were carried out in accordance with the

Declaration of Helsinki and approved by the research ethics board of the University of Leipzig (EK-No.: 246—2009—09112009).

Following the MRI acquisition at each scan session, fasting blood samples were taken to determine serum hormone levels of estrogen, progesterone, LH, and cortisol. Blood samples were analyzed at the Institute for Laboratory Medicine of the University Hospital Leipzig by the fully automated Roche Cobas® system (Roche, Basel, Switzerland). All samples were measured within one plate; intra-assay coefficients of variance (CV) were within 3.2–6% for estrogen, 2.3–5.2% for progesterone, 1.6–2% for LH and 2.1–5.9% for cortisol. Menstrual cycles were further monitored by LH surge ovulation tests (Diagnostik Nord GmbH hLH-K20 hLH Kassettentest).

In order to detect any potential influence that mood or stress levels may have on resting state connectivity, the following scales were administered prior to every scan: the Visual Analog Scale (VAS) (Grant et al., 1999), the Profile of Mood states (PoMS) (Pollock et al., 1979), the Perceived Stress Scale (PSS) (Cohen et al., 1983), and a standardized assessment of implicit positive and negative affect (IPANAT) (Quirin et al., 2009).

MRI DATA ACQUISITION

Imaging was performed each second or third day across four menstrual cycles: each session was started at a different menstrual cycle day to control for any potential scanner-drift artifacts. Thus, each scanning session provides data for one full menstrual cycle scanned across two menstrual cycles (session 1: January 2012, session 2: November 2012), resulting in 16 scans per session and a total of 32 longitudinal scans of the same individual. To control for circadian rhythm effects, every scan was collected at the same time of the day (7:30 am).

All 32 MRI sessions were performed on a 3-Tesla Magnetom Verio scanner (Siemens, Erlangen, Germany) equipped with a 32-channel head array coil. In each session, resting-state fMRI data were acquired using a gradient-echo echo-planar imaging (EPI) sequence. The subject was asked to stay awake, relax, and look at a low-contrast fixation cross. The following parameters were used: 300 whole brain volumes, acquisition matrix = 64 × 64, slice thickness = 4 mm (0.8 mm gap), resulting in a nominal voxel size of $3 \times 3 \times 4.8\,mm^3$. Further imaging parameters: 30 axial slices, TR = 2000 ms, TE = 30 ms, flip angle = 90° and bandwidth = 1954 Hz/pixel. The total scanning time for resting-state fMRI was 10 min.

In addition, for the purpose of image registration and normalization, high-resolution T1-weighted images were acquired using a three-dimensional Magnetization-Prepared Rapid Gradient Echo (MPRAGE) sequence. The Alzheimer's Disease Neuroimaging Initiative (ADNI) standard protocol was used with the following parameters: TI 900 ms, TR 2300 ms, TE 2.98 ms, flip angle 9°, band width 238 Hz/pixel, image matrix 256 × 240, 176 partitions, FOV $256 \times 240 \times 176\,mm^3$, sagittal orientation, average voxel size $1 \times 1 \times 1\,mm^3$.

DATA ANALYSIS

Preprocessing of all 32 resting-state fMRI data sets was performed using SPM8 (Wellcome Department of Imaging Neuroscience, London, UK) rev. 8.4010 using (Matlab™ 7.11) including estimation and correction for motion and EPI deformation. Functional images were then co-registered with the high-resolution anatomical image, and normalization was performed using the unified segmentation approach (Ashburner and Friston, 1997). Following normalization, the resulting voxel size of the functional images was interpolated to an isotropic voxel size of $3 \times 3 \times 3\,mm^3$. In the final step of the preprocessing, the functional images were spatially smoothed with a Gaussian kernel of 8 mm full width at half maximum (FWHM).

To identify cycle-dependent connectivity changes, EC mapping (Lohmann et al., 2010) was performed using the LIPSIA software package (Lohmann et al., 2001). EC is able to detect central hubs within a brain network using an algorithm similar to Google's PageRank algorithm, which highlights websites most often linked to other highly interconnected websites (Brin and Page, 1998). Similarly, we were interested in brain regions of high individual connectivity that are, in addition, strongly connected to other regions of high connectivity (i.e., functional brain-hubs). Using EC mapping, we investigated potential correlations between cycle-dependent sex hormone changes and intrinsic functional connectivity. To restrict the EC analysis to meaningful gray matter regions, a mask was generated using the gray matter segmentation of the subject's high-resolution T1-weighted image. This gray matter segmentation was smoothed with 4 mm FWHM, and voxels showing a minimum gray matter probability of 0.12 were included in subsequent EC analysis. For all voxels within this mask, a similarity matrix was generated including Pearson's correlation coefficient between all resting-state fMRI time courses. In order to use a similarity matrix with only positive numbers, a value of 1 was added to all matrix entries before computing the EC. According to the theorem of Perron and Frobenius, this similarity matrix has a unique real largest eigenvalue, and the corresponding eigenvector has strictly positive components (Perron, 1907). Then the EC map was generated using the components of this eigenvector to determine the EC of all voxels.

For each resting-state measurement, EC analysis was computed separately resulting in a set of 32 EC maps. Statistical analysis was performed with SPM8 based on the general linear model using a factorial design. The design matrix was build using columns coding both scanning periods and further covariates including ovarian hormone and cortisol levels. A contrast was specified in order to test for positive or negative correlations between hormones and EC. The resulting statistical parametric maps were processed using a voxel-wise threshold of $p < 0.001$, and remaining clusters were shown with correction for multiple comparisons using family-wise error (FWE) correction at $p < 0.05$. FWE (family wise error) correction for multiple comparisons was applied correcting for $n = 575$ (the number of resells = resolution elements that remain after smoothing the image of the whole brain that contained 61940 voxels in total).

EC mapping is a measure of the interconnectedness of a brain region with all other nodes. As it does not explain whether connectivity changes are specific to another brain region, or reflect a regionally unspecific and global change in connectivity, we performed seed-based correlation analyses. To this end, we placed seed-regions in the left and right DLPFC, and in the left and right

sensorimotor cortex. We fed the resulting correlation maps into a statistical analysis using the general linear model and included progesterone level as a covariate in the design matrix. A contrast was specified in order to test for a positive correlation between progesterone levels and connectivity changes obtained by the seed-based correlation analyses. The resulting statistical parametric maps were processed using voxel-wise thresholds of $p < 0.005$ and $p < 0.001$, and an extent threshold of $k > 20$ voxels.

RESULTS

HORMONE ASSESSMENT

Hormone analyses confirmed characteristic patterns for estrogen and progesterone across all menstrual cycles assessed (for details, see **Figure 1**).

EC MAPPING- WHOLE BRAIN ANALYSIS

EC attributes a centrality value to each voxel in the brain such that a voxel receives a larger value if it is more strongly correlated with many other voxels, which are central within the network themselves. We found progesterone levels to modulate intrinsic functional connectivity across the entire brain, i.e., higher progesterone levels were associated with increased EC values in bilateral sensorimotor cortex and in the right dorsolateral prefrontal cortex (DLPFC) (**Figures 2**, **3**, left panel; details of the analysis are provided in **Table 1**). We also found a cluster in the left DLPFC that did not survive the FWE correction. However, we report this cluster due to symmetry reasons. For whole brain analyses, cortisol was used as a covariate of no interest (for analysis-details, see Methods-Section). Neither positive nor negative correlations were found with estrogen.

SEED-BASED ANALYSES

Seed based correlation analyses were used to find changes in BOLD signal correlations from centrality clusters to other areas across the brain. Our seed based correlation analyses revealed EC changes in right (and left) DLPFC (**Figure 2**, right panel) as well as bilateral sensorimotor cortex (**Figure 3**, right panel) to bilateral hippocampus to correlate with progesterone levels.

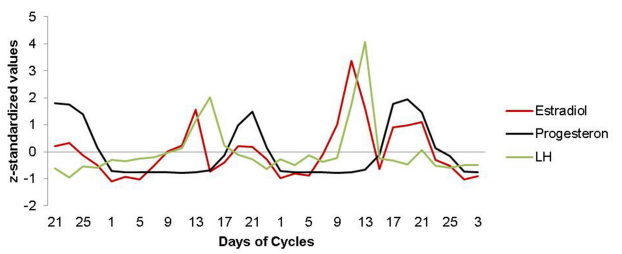

FIGURE 1 | Estradiol, progesterone and LH fluctuation across the menstrual cycle. Characteristic patterns of serum estradiol (red line), progesterone (black line) and LH (green line) levels are displayed across two menstrual cycles. The depicted data represents days of cycle with corresponding z-standardized, single hormone values, matching the single scan time points in chronological order. As expected, estradiol shows a first prominent peak in the periovulatory phase, followed by a second peak in the late luteal phase. LH surges after the peak of estrogen, shortly before ovulation. Progesterone levels are low during the follicular phase and high during the luteal phase.

We interpret these results as high progesterone to be paralleled with high connectivity between right DLPFC, bilateral sensorimotor cortex and hippocampus. For the left DLPFC (**Figure 2**, top panel), connectivity-changes to bilateral hippocampus were observed at a voxel-wise threshold of $p < 0.005$ (unc.), while the changes for the right DLPFC were significant at both voxel-wise thresholds $p < 0.001$ and $p < 0.005$ (unc.). In the seed based analysis of bilateral sensorimotor cortex (**Figure 3**, right panel), progesterone-modulated intrinsic connectivity-changes in the right and left sensorimotor cortex were found to connect with the left hippocampus at a voxel-wise threshold of $p < 0.001$ (unc.), and a bilateral effect with both hippocampi was demonstrated at $p < 0.005$ (unc.).

DISCUSSION

In the present study, we report the effects of the menstrual cycle on intrinsic connectivity in a single female subject, using a longitudinal design. In the initial whole-brain EC analysis, we find an FWE-corrected correlation of progesterone-levels with intrinsic connectivity changes in right DLPFC and bilateral sensorimotor cortex. Without FWE-correction, we also found a cluster in the left DLPFC that we also report due to symmetry aspects. Subsequent seed-based analyses demonstrate these progesterone-modulated intrinsic connectivity changes in right and left DLPFC connect to the bilateral hippocampus. We also observe a progesterone-modulated intrinsic connectivity change in bilateral sensorimotor cortex with the bilateral hippocampus.

A large body of evidence indicates a crucial role for the DLPFC in attention-demanding cognitive tasks involving domains of working memory and executive function (Curtis and D'Esposito, 2003), decision-making (Heekeren et al., 2006), reasoning (Goel and Dolan, 2004), problem solving (Ruh et al., 2012) and emotion-regulation (Levesque et al., 2003). The majority of treatment studies applying transcranial magnetic stimulation in unipolar depression target the DLPFC (Paus and Barrett, 2004; Padberg and George, 2009) for improved regulation of affective states, and enhanced cognitive control over stress and emotion responsiveness (Davidson et al., 2002). An essential feature of the DLPFC, which allows it to serve as a gate-keeper role across multiple neural networks, is its extensive interconnectedness with other brain regions, including thalamus, basal ganglia, orbitofrontal cortex, primary and secondary association areas of neocortex (Tekin and Cummings, 2002; Dosenbach et al., 2007) and hippocampus (Meyer-Lindenberg and Weinberger, 2006; Bilek et al., 2013). Dense pathways connect the DLPFC and the hippocampal formation (Goldman-Rakic et al., 1984), both directly and indirectly, and interactions between these regions are implicated in episodic memory (Simons and Spiers, 2003), the regulation of emotional–motivational states (Phillips et al., 2003), and experience-dependent plasticity (Bilek et al., 2013). Prefrontal (Brinton et al., 2008), and hippocampal (McEwen, 2002) regions are also exceptionally rich in the expression of sex steroid receptors. Evidence from animal work suggests sex hormones to be potent modulators of synaptic growth and density in regions of the prefrontal cortex and hippocampus (McEwen, 2002; Sanz et al., 2008; Galea et al., 2013). These findings are extended by human neuroimaging data that link changes in sex hormone

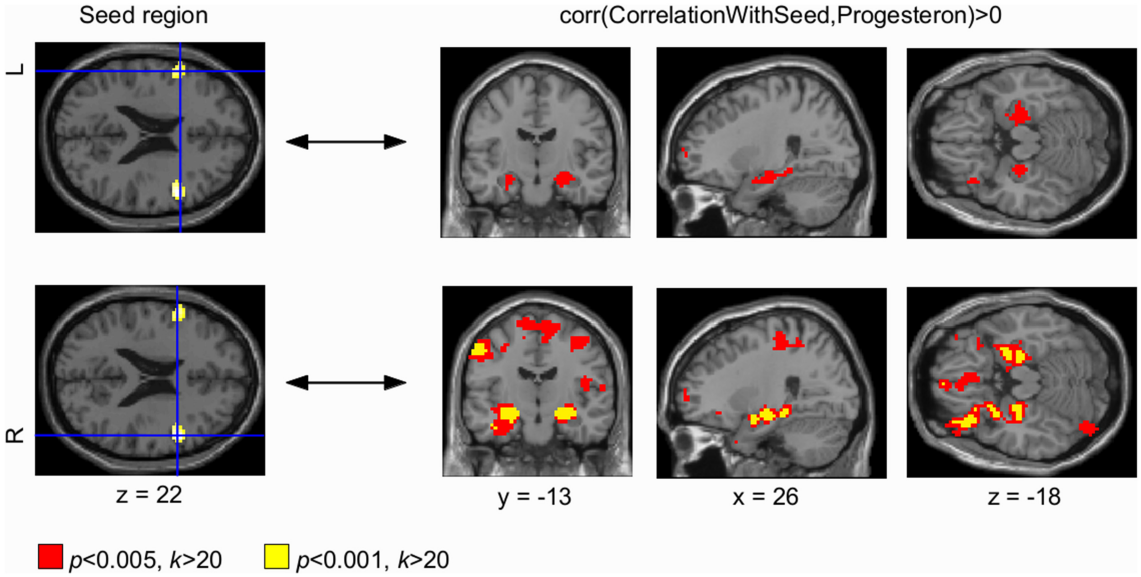

FIGURE 2 | Progesterone-modulated functional connectivity in bilateral dorsolateral prefrontal cortex and hippocampus. The whole brain Eigenvector Centrality Mapping (EC) analysis reveals a significant correlation of progesterone with bilateral dorsolateral prefrontal cortex (DLPFC) ($p < 0.001$ unc.; **left panel**). In the seed based analysis, these progesterone-modulated intrinsic connectivity-changes in right and left DLPFC were found to connect with bilateral hippocampus **(right panel)**. For the left DLPFC (top panel), connectivity-changes to bilateral hippocampus were observed at a threshold of $p < 0.005$ (signal change depicted in red). The right DLPFC also showed progesterone-modulated connectivity with bilateral hippocampus modulated by progesterone at both thresholds: $p < 0.001$ (unc.; signal change depicted in yellow) and $p < 0.005$ (unc.; signal change depicted in red).

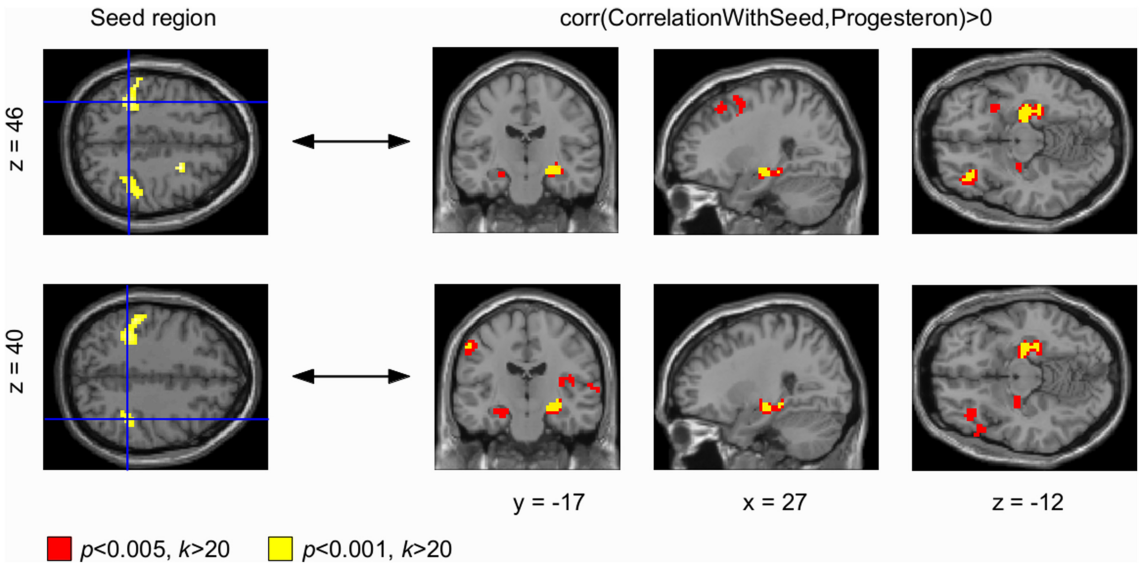

FIGURE 3 | Progesterone-modulated functional connectivity in bilateral sensorimotor cortex and hippocampus. The whole brain Eigenvector Centrality Mapping (EC) analysis reveals a significant correlation of progesterone with bilateral sensorimotor cortex ($p < 0.001$ unc.; **left panel**). In the seed based analysis **(right panel)**, these progesterone-modulated intrinsic connectivity-changes in right and left sensorimotor cortex were found to connect with the left hippocampus (signal change depicted in yellow) at a threshold of $p < 0.001$ (unc.); for $p < 0.005$ (unc.) a bilateral effect with both hippocampi could be demonstrated (signal change depicted in red).

levels with neural activity patterns in the above referenced regions during cognitive and affective tasks (Goldstein et al., 2005, 2010; Andreano and Cahill, 2010; Frank et al., 2010; Bayer et al., 2014). While systematic structural imaging studies exploring the effects of the menstrual cycle are still sparse, the limited evidence that has been collected comparing scans during the follicular and the luteal phase, or using between-group designs, support cycle-phase related changes in frontal cortical regions (Pletzer et al., 2010) and

Table 1 | Overview on detailed statistics (coordinates, cluster size, *p*-values) of results.

Set level		ClusterLevel				PeakLevel							
P	***c***	$p_{FWEcorr}$	$\alpha_{FDRcorr}$	k_E	p_{uncorr}	$p_{FWEcorr}$	$\alpha_{FDRcorr}$	***t***	***Z***	p_{uncorr}	**mm**	**mm**	**mm**
0.000	7	0.040	0.019	73	0.004	0.338	0.531	5.11	4.19	0.000	51	8	22
		0.548	0.156	23	0.078	0.538	0.531	4.80	4.00	0.000	24	5	46
		0.000	0.001	185	0.000	0.663	0.531	4.63	3.90	0.000	−51	−34	43
						0.708	0.531	4.57	3.86	0.000	−63	−31	31
						0.786	0.531	4.46	3.79	0.000	−36	−43	46
		0.191	0.058	43	0.021	0.752	0.531	4.51	3.82	0.000	−57	11	22
		0.101	0.037	55	0.010	0.986	0.738	3.93	3.44	0.000	57	−61	−5
		0.345	0.097	32	0.042	0.990	0.738	3.89	3.40	0.000	−21	−67	55
						0.993	0.738	3.85	3.38	0.000	−12	−73	58
		0.008	0.005	108	0.001	0.992	0.738	3.87	3.39	0.000	48	−34	46
						0.992	0.738	3.86	3.39	0.000	39	−43	40
						0.999	0.911	3.62	3.22	0.001	30	−46	52

Table shows 3 local maxima more than 8.0 mm apart, p-values adjusted for search volume.
Height threshold: T = 3.34, p = 0.001 (1.000).
Extent threshold: k = 20 voxels, p = 0.098 (0.630).
Expected voxels per cluster, <k> = 7.511.
Expected number of clusters, ‹c›= 1.00.
FWEp: 6.123, FDRp: Inf, FWEc: 73, FDRc: 55.
Degrees of freedom = [1.0, 25.0].
FWHM = 13.5 13.1 13.0 mm mm mm; 4.5 4.4 4.3 {voxels}.
Volume: 1672380 = 61940 voxels = 575.3 resels.
Voxel size: 3.0 3.0 3.0 mm mm mm; (resel = 85.56 voxels).

hippocampus (Protopopescu et al., 2008). Petersen et al. (2014) investigated intrinsic functional connectivity changes in users of oral contraceptives and compared the results to naturally cycling women (Petersen et al., 2014). The authors used ICA to evaluate the differences in resting state activity between these two groups. Of 20 compounds the analysis produced, 11 were discarded as likely artifactual findings and nine were retained (Petersen et al., 2014). These were identified amongst others as somatosensory, left frontoparietal, right frontoparietal, posterior default mode, anterior default mode and executive control networks. The anterior default mode and executive control network were selected as the networks of interest. Altered resting state dynamics could be shown in both networks, specifically the connectivity of the left angular gyrus, the left middle frontal gyrus and the anterior cingulate cortex were different between groups (Petersen et al., 2014). Luteal women showed reduced coherence between left angular gyrus and the rest of aDMN compared to follicular women. In the executive control network luteal women showed reduced coherence between right anterior cingulate and the rest of the network when compared to follicular women (Petersen et al., 2014). Users of oral contraception in the active pill phase showed reduced coherence between left anterior cingulate and the rest of the network as well as reduced coherence between the left middle frontal gyrus and the executive control network compared to naturally-cycling women in the follicular phase (Petersen et al., 2014). Our findings, particularly intrinsic connectivity changes in left DLPFC, partly correspond to these results, even though we found a specific modulation by progesterone and we did not analyze women on oral contraception as Petersen et al. found changes in connectivity in the left middle frontal gyrus, which is part of DLPFC, for women in the active pill phase compared to women in the follicular phase. Additional changes of intrinsic connectivity that we found, especially in sensorimotor cortex, may differ due to methodological reasons: limitation of Petersen et al. to the described two networks as well as the different analytical approach (ICA vs. EC). However, both studies show that there are changes of intrinsic connectivity during the menstrual cycle and provide evidence that endogenous hormones influence the baseline state of the brain (Petersen et al., 2014).

Our findings of progesterone-modulated intrinsic connectivity changes in the DLPFC and hippocampus are consistent with these converging lines of evidence from animal and human data that suggest the menstrual cycle influences structural and functional connectivity in sex-hormone receptor rich brain areas.

We also observed progesterone-modulated connectivity changes in the bilateral sensorimotor cortex in our whole brain analysis that revealed specific hippocampal connections in the subsequent seed-based analyses. These findings could be interpreted as menstrual cycle specific changes in hippocampal modulation of sensorimotor processes given that the hippocampus combines I) sensory afferents and synaptic mechanisms underlying rapid learning, as well as II) links to motivational, emotional, executive and sensorimotor functions (Bast, 2007). Specific to the menstrual cycle, it has been consistently reported that differences in pain perception are modulated by endogenous hormonal fluctuations (Riley et al., 1999), including areas within the sensorimotor cortex (Veldhuijzen et al., 2013). In women suffering from dysmenorrhea (severe menstrual pain), trait

(Tu et al., 2010)- and state (Tu et al., 2013)-related gray matter changes were found in regions associated with pain modulation, pain transmission, and affective experience generation. Their findings include atrophic gray matter changes in the left secondary somatosensory cortex and hypertrophic changes in the primary somatosensory cortex (Tu et al., 2010, 2013), regions involved in sensory discrimination and interoception (Craig, 2002). Consistent with these results, our findings provide evidence for menstrual cycle modulated changes in functional connectivity in the somatosensory system.

The primary aim of the current study was to test the feasibility of extensive multiple longitudinal scanning sessions across the menstrual cycle for the resting state modality. A crucial limitation of the present longitudinal study was that it was conducted in a single subject. We acknowledge the limitations from this single-case design and the preliminary nature of our dataset. Our results have to be interpreted with caution and warrant replication in a larger sample. The current findings support the feasibility of combining rigorous menstrual monitoring and longitudinal, short-interval, intra-individual fMRI scanning protocols and thus, serve as a proof-of-principle study.

A portion of our findings, such as the EC changes in DLPFC, are consistent with the one study thus far (Petersen et al., 2014) that has reported functional connectivity changes across the menstrual cycle. However, Petersen et al did not show any significant association between either progesterone or estrogen levels with the connectivity changes observed in the aDMN or the ECN. Several methodological aspects could contribute to these controversial findings: Petersen et al. (2014) only assessed sex hormone levels at two different time-points during the menstrual cycle, once in the follicular phase and once in the luteal phase. As progesterone levels are only substantially elevated in the luteal phase, the variability in progesterone levels might not have been wide enough to capture any significant effects in a correlation analysis. While we have applied a whole brain approach, limiting the analysis to specific networks may have also affected their sex hormone correlation analyses.

We further acknowledge that the graph-based parameter EC measure is one that can be viewed as difficult to relate to brain function. Expert recommendations (Buckner et al., 2013; Sporns, 2014) have repeatedly stated that, while intrinsic functional connectivity provides a powerful and unique tool to provide insight into human brain organization, this method is also based on inherently ambiguous measures and limited by constraints from static anatomical connectivity and from poorly understood dynamic functional coupling changes. Thus, reports from resting-state studies are best interpreted in the context of external experiments. The converging evidence from animal and human literature (McEwen, 2002; Protopopescu et al., 2008; Galea et al., 2013), which suggest that the hippocampus is among the most susceptible of brain regions to subtle hormonal fluctuation, support our findings that link menstrual cycle phase to individual functional connectivity in this region.

We did not acquire perfusion data in our experiments, thus we are not able to directly test whether the EC changes we observed were influenced by blood flow. However, to date there is one study published that investigated perfusion changes in the female brain across the menstrual cycle: the authors report no cyclic variation in resting-perfusion using continuous arterial spin labeling (ASL) (Ances and Detre, 2003). Five female subjects were investigated at two times during their menstrual cycle: the initial scan was performed at the late luteal phase (1 or 2 days prior to the start of menses) and the second scan 4–5 days after completion of the menses (mid-follicular period). No significant differences in resting perfusion were observed for these two phases of the menstrual cycle. Liang et al. (2014) investigated cerebral blood flow (CBF) networks using ASL. The authors showed that highly connected brain regions overlap mostly with hub regions detected by BOLD fMRI studies. As expected, hub regions measured by ASL were characterized by shorter characteristic path length but higher vulnerability and eigencentrality. A positive nonlinear relationship of EC to regional CBF was shown. We cannot exclude that EC in general might be somewhat sensitive to perfusion changes. However, so far no evidence points to menstrual cycle specific perfusion changes and thus, we think it is unlikely that perfusion changes alone underlie the pattern of menstrual cycle specific EC-changes we report here.

In summary, our results suggest that the menstrual cycle substantially impacts intrinsic functional connectivity, particularly in brain areas associated with contextual memory-regulation, such as the hippocampus. We also observe progesterone-modulated changes in functional connectivity in bilateral DLPFC and sensorimotor cortex, regions that have been implicated in emotional regulation and pain modulation, domains that have previously been identified to be susceptible to menstrual cycle dependent rhythms. These results argue that the menstrual cycle is an important factor to consider when studying short-term functional plasticity in the human brain and highlight the importance of controlling for menstrual cycle phase in neuroimaging studies.

It is important to note that our data is of preliminary nature and warrants replication in a larger sample size. This longitudinal, single-subject study provides a first step toward the development of more individualized strategies of mapping important modulators of human brain states, such as the menstrual cycle. We applied rigorous menstrual monitoring and demonstrate the feasibility of longitudinal, short-interval, intra-individual fMRI scanning in combination with a simple analysis measure for whole brain intrinsic connectivity, which is what makes this pilot study stand out as a proof of principle. The current preliminary findings contribute to the development of more individualized mapping-strategies of the human brain by integrating potential mediators of brain states, such as menstrual cycle phase.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2015.00044/abstract

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How to be patient. The ability to wait for a reward depends on menstrual cycle phase and feedback-related activity

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Dopamine (DA) plays a major role in reinforcement learning with increases promoting reward sensitivity (*Go learning*) while decreases facilitate the avoidance of negative outcomes (*NoGo learning*). This is also reflected in adaptations of response time: higher levels of DA enhance speeding up to get a reward, whereas lower levels favor slowing down. The steroid hormones estradiol and progesterone have been shown to modulate dopaminergic tone. Here, we tested 14 women twice during their menstrual cycle, during the follicular (FP) and the luteal phase (LP), applying functional magnetic resonance imaging while they performed a feedback learning task. Subsequent behavioral testing assessed response time preferences with a clock task, in which subjects had to explore the optimal response time (RT) to maximize reward. In the FP subjects displayed a greater learning-related change of their RT than during the LP, when they were required to slow down. Final RTs in the slow condition were also predicted by feedback-related brain activation, but only in the FP. Increased activation of the inferior frontal junction and rostral cingulate zone was thereby predictive of slower and thus better adapted final RTs. Conversely, final RT was faster and less optimal for reward maximization if activation in the ventromedial prefrontal cortex was enhanced. These findings show that hormonal shifts across the menstrual cycle affect adaptation of response speed during reward acquisition with higher RT adjustment in the FP in the condition that requires slowing down. Since high estradiol levels during the FP increase synaptic DA levels, this conforms well to our hypothesis that estradiol supports *Go learning* at the expense of *NoGo learning*. Brain-behavior correlations further indicated that the compensatory capacity to counteract the follicular *Go bias* may be linked to the ability to more effectively monitor action outcomes and suppress bottom-up reward desiring during feedback processing.

Keywords: menstrual cycle, estradiol, dopamine, time perception, fMRI, RCZ, VMPFC, reinforcement learning

INTRODUCTION

Time estimation is an essential perceptual skill involved in a variety of behavioral aspects. It influences our decision making in every-day life such as which line to choose at the supermarket based on previous experiences, but also plays a role in motor control (e.g., speed of speech or movement). As to the mechanism underlying time perception in the seconds-to-minutes range, also known as interval timing, a model of an internal clock has been proposed. According to this model a pacemaker sends pulses representing units of elapsed time to a counter, which thereby obtains an estimate of a time duration (Treisman, 1963). However, the speed of this pacemaker is prone to outer influences and thus only reflects a subjective and possibly inaccurate perception of time. More precisely, a higher frequency of sent pulses by the pacemaker results in an overestimation of passed time. For instance, an emotionally arousing context or stimulating substances such as cocaine or methamphetamine may increase the speed of the inner clock (Maricq and Church, 1983; Matell et al., 2004; Droit-Volet and Gil, 2009). Likewise, evidence from animal studies indicates an important role of the steroid hormone 17ß-estradiol (E2) in the modulation of time perception. Accordingly, ovariectomized rodents displayed enhanced internal clock speed after E2 injection (Sandstrom, 2007; Pleil et al., 2011). However, E2 may not directly modulate internal clock speed, but its effect on time perception may rather be a function of the interaction between E2 and dopamine (DA), a neurotransmitter that has also been implicated in time estimation (Rammsayer, 1993; Meck et al., 1996; Buhusi and Meck, 2005). This idea is also supported by other rodent studies reporting a modulating role of E2 on mesolimbic dopaminergic transmission. E2 thereby enhanced dopaminergic tone, on the one hand by stimulating DA release and DA synthesis rate and on the other hand by increasing the density of DA D1 receptors and downregulating D2 receptors (Lévesque et al., 1989; Becker, 1999). In contrast to that, the neurosteroid progesterone (PROG) opposes these effects. PROG has been shown to diminish E2 receptor density, to stimulate enzymes involved in the degradation of DA, and to act positively on GABA receptors, thereby inhibiting dopaminergic neurons, all of which leading to

a reduced dopaminergic tone (Luine and Rhodes, 1983; Dluzen and Ramirez, 1984; Majewska et al., 1986; Mauvais-Jarvis et al., 1986). In that way, it may be hypothesized that these neurohormones should exert contrasting effects on the perception of time by increasing vs. reducing internal clock speed.

DA has been proposed to act on action selection via a direct "Go" or an indirect "NoGo" projection pathway from the basal ganglia to the cortex by either facilitating responses or by inhibiting them respectively (Frank et al., 2004). Both, the D1 and D2 receptor type, are integrated in these pathways and stimulated differently by prevalent DA levels. Frank et al. (2004) found that during a reinforcement learning task Parkinson patients being off medication, who thus suffered from depleted DA levels, showed an impaired ability to learn via the "Go" pathway to choose a rewarded stimulus. At the same time, they displayed increased punishment sensitivity and were more able to avoid stimuli that would have led to a negative feedback (i.e., they exhibited better learning via the "NoGo" pathway). The authors proposed that the DA bursts signaling a reward, facilitate learning via positive reinforcement by acting on D1 receptors in the "Go" pathway. On the other hand dips in DA levels caused by negative feedback stimulate the D2 receptor type, which is implemented in the indirect "NoGo" pathway (Frank et al., 2004). This principle has also been shown to transfer to the response time domain: Parkinson patients on medication (i.e., with normal DA levels) were better in learning to adapt their response time to a high response speed in order to maximize their reward. However, when being off medication (i.e., decreased DA levels) they showed better performance in a condition, in which they had to slow down and wait to get the highest reward (Moustafa et al., 2008). Hence, the adaptation of response speed in the context of maximizing reward value appears to be also regulated by the two different pathways, in that the "Go" pathway favors speeding up and the "NoGo" pathway facilitates slowing down.

In our study, we wanted to investigate (1) whether the preference for "Go" over "NoGo" learning in a response time adjustment paradigm depends on naturally varying levels of E2 and PROG during the menstrual cycle and (2) whether the ability to wait for a reward depends on neural responses during reinforcement learning. For this purpose, we tested healthy female subjects twice during their menstrual cycle: once during the late follicular phase (FP), in which E2 levels are high and PROG levels low, and a second time during the mid luteal phase (LP) that is dominated by increased PROG levels. On both test days, subjects underwent functional magnetic resonance imaging (fMRI) while performing a probabilistic feedback learning task. Following this, a response time adjustment paradigm (the clock task) was conducted, in which subjects had to explore the optimal response time (RT) for reward maximization. The behavioral data from the clock task were then analyzed in relation to feedback-related brain activity from the probabilistic learning task. By this we intended to establish the link between the ability to optimize response speed in the clock task (e.g., optimized reaction time as a subject-specific behavioral parameter) and the neural correlates implicated in mediating the preference for "Go" over "NoGo" learning and vice versa. This procedure is similar to the one employed by Hariri et al. (2006). In this study the behavioral parameter obtained from a delay discounting task conducted outside of the scanner (i.e., the subject-specific delay discounting parameter k) correlated with reward-related activity in the ventral striatum assessed via fMRI. In the behavioral delay discounting task, subjects were given the option to choose between a smaller immediate reward and a higher reward that was to be obtained later in time and thus had to be waited for (i.e., the "delayed reward"). The delay discounting task bears some resemblance to the clock task, since it requires an estimation of time combined with an associated reward value. Hence, we chose to follow the approach used by Hariri et al. (2006) in order to investigate if feedback-related neural responses correlated with post-scan response time adjustment parameters.

Based on the above mentioned findings, we predicted preferences for Go over NoGo learning observed in the probabilistic learning task to correlate with the favored learning style in the clock task. Individuals who were prone to learning via positive reinforcement (Go learning) were expected to display a greater ability to adapt their RT to a high speed for reward maximization. In contrast to that, those learning better to avoid punished options were expected to more easily adjust RTs to a slow pace and to wait for a higher reward. As to a possible effect of menstrual cycle, we hypothesized that particularly during the FP, in the presence of high E2 levels, subjects should have difficulties to be patient and to wait for the highest possible reward in a condition that requires slowing down. On the neural level, we further predicted correlations between RT adaptation in the clock task and feedback-related activity in regions of the mesolimbic dopamine system. In particular, the rostral cingulate zone (RCZ) has been shown to be involved in learning from errors and negative feedback (NoGo learning) (e.g., Klein et al., 2007b; Jocham et al., 2009). This led to our assumption that differences in RCZ activity during negative feedback should be predictive of RT adaptations in the clock task across the menstrual cycle, especially when subjects had to wait for a higher reward (i.e., the SLOW condition). Additionally, we expected opposite correlations with RT adaptation in regions that have been associated with reward valuation and positive reinforcement learning such as the ventromedial prefrontal cortex (VMPFC) (see Kringelbach and Rolls, 2004; McClure et al., 2004; Fellows, 2007; Hare et al., 2008; Grabenhorst and Rolls, 2011; Jocham et al., 2011; Peters and Büchel, 2011 for overview).

MATERIALS AND METHODS

SUBJECTS

Fourteen healthy female subjects (mean age ± SD: 24.9 ± 1.6; age range: 22–28 years) participated in the fMRI and the behavioral study twice during their menstrual cycle. All subjects were right-handed and had no history of psychiatric, neurological, or hormonal illnesses or other forms of chronic disease (e.g., diabetes). Further exclusion criteria were the use of any medication on a regular basis or of hormonal contraceptives. Subjects were also screened for a regular menstrual cycle with an average cycle length between 26 and 32 days (mean cycle length ± SD: 29.8 ± 2.7).

All subjects gave written informed consent and were paid for participation. This study was approved by the local

ethics committee of the medical association of Hamburg (Aerztekammer Hamburg).

CYCLE MONITORING AND SALIVA ANALYSIS

In this experiment, we applied a counter-balanced within-subject design. Each subject was tested twice during her menstrual cycle: once in the late follicular phase (FP) and a second time in the mid luteal phase (LP). To determine the right time point for the two test sessions, subjects were asked to monitor their cycle with the Clearblue Fertility Monitor® and to inform the experimenters about the displayed details. Test days were then arranged individually according to the course of the subject's cycle. On average (mean ± SD), the follicular test took place on day 13.3 ± 2.3 of a regular cycle, 3.9 ± 1.6 days before the Lutropin (LH) surge triggered ovulation. The luteal test took place on day 24.1 ± 2.0, 7.3 ± 2.8 days after the LH surge and 5.4 ± 2.1 days before the next cycle began.

In addition, morning saliva samples were collected at the 2 days of testing and the day following the onset of menstruation. This was done to ensure that the two sessions were in fact set in the targeted cycle phases by analyzing concentrations of free, bioactive E2 and PROG. A second reason for assessing hormonal parameters was the fact that young women still undergo anovulatory cycles occasionally. Thus, only subjects showing a post-ovulatory increase in PROG in the LP relative to the FP, which is a reliable sign of successful ovulation, were included in the analyses. A 17beta-Estradiol Luminescence Immunoassay and a Progesterone Luminescence Immunoassay purchasable from IBL International were used to determine E2 and PROG concentrations following the standard procedure described in the manual. The sensitivity of the 17beta-Estradiol kit is denoted to be 0.3 pg/mL at the 2 SD confidence limit and for the progesterone kit 2.6 pg/mL.

All 14 subjects showed increased PROG levels in the LP compared to the FP (salivary PROG concentration [mean ± SD]: FP = 51.65 ± 34.97 pg/mL; LP = 141.40 ± 94.41 pg/mL). Conversely, mean E2 levels were higher during FP than during LP (salivary E2 concentration [mean ± SD]: FP = 4.56 ± 3.84 pg/mL; LP = 4.02 ± 2.59 pg/mL).

THE CLOCK TASK

Subjects performed a response time adjustment paradigm (modified from Moustafa et al., 2008) outside of the scanner, which we called the "clock task." In this task, subjects were presented with three different running clock faces indicated by different colors, and were instructed to explore at which point in time they had to press a stop button in order to maximize personal reward. Reward value, as a function of response time (RT) during a full clock-arm turn of 5 s, varied between the different clocks. For one clock fast responses yielded highest reward (FAST clock favoring Go learning). In contrast, a second clock required patience, meaning that subjects had to delay responding to maximize reward (SLOW clock favoring NoGo learning). The exact reward value of each trial in the FAST and the SLOW clock condition was calculated with a cosine function, but always ranged between a minimum of 15 and a maximum of 60 (see **Figure 1**). The third clock acted as a control condition with no contingency between response time and magnitude of reward outcome (RANDOM clock, in which points varied randomly across given response times). Exact reward values in the RANDOM condition were determined by multiplying a random number with the difference between the maximum and minimum reward value (i.e., 60–15) and adding the minimum reward value to this product. In all three clock conditions, a random noise parameter (range: −5 to +4 points) was added to the calculated reward value in order to prevent subjects to associate a specific RT with a certain reward value. After each response immediate reward feedback was provided. Feedback was always followed by a blank screen that lasted the residual time that would have been necessary to finish the whole clock arm turn before the next trial began. If no response was given within a full clock arm turn (after 5 s) no points were won and subjects had to wait a period of another 5 s before the next clock face was shown. Each of the three clock types (FAST, SLOW, and RANDOM) was presented 50 times in a row. Between these runs (i.e., 50 trials of one clock type) there was a short break. During this break subjects were informed that they would be confronted with a new type of clock in the following run and were again instructed to figure out the optimal response time. The new run was started as soon as the subject indicated to be prepared. The sequence of the runs for the three clock types was counterbalanced across participants and cycle phases.

PROBABILISTIC LEARNING TASK

Subjects underwent fMRI while performing a reinforcement learning task. This task was adapted from previous studies showing that performance in this learning task depends on dopaminergic transmission (Frank et al., 2004; Klein et al., 2007b). Subjects were presented with three different pairs of arbitrary symbols and had to learn which symbol was the most rewarded option. For that, they received probabilistic feedback in form of a smiley or a grumpy after each choice. The three symbol pairs—called "AB," "CD," and "EF"—varied in their probability for positive or negative feedback: in the stimulus pair "AB" a choice of "A" yielded positive feedback in 80% of the cases and led to negative feedback in 20%. Choosing "B," on the other hand, led to a rewarding smiley in only 20% of the cases and to negative feedback with a probability of 80%. The other symbol pairs yielded positive or negative feedback in a corresponding probabilistic manner with a choice of "C" of the pair "CD" being rewarded in 70% and "D" in 30% of the cases. A choice of "E" of the pair "EF" yielded positive feedback with a probability of 60%, whereas "F" was rewarded in 40% of the cases. Thus, in the course of this task, subjects should have either learned that choosing "A" was the most rewarded option or that choosing "B" was the most punished option, depending on their preferences for Go or NoGo learning. To test for learning success, subjects had to complete a second session outside the scanner, in which the symbols were mixed in new pairs ("AC", "AD", "AE", "AF", "BC", "BD", "BE", "BF", "CD", "CE", "CF", "DE", "DF"). Individuals who learned better via reward (Go learning) should display a higher tendency to choose the symbol "A" from these new stimulus pairs, whereas individuals that learned better via punishment (NoGo learning) should tend to avoid "B" in the pairs including the symbol "B." For

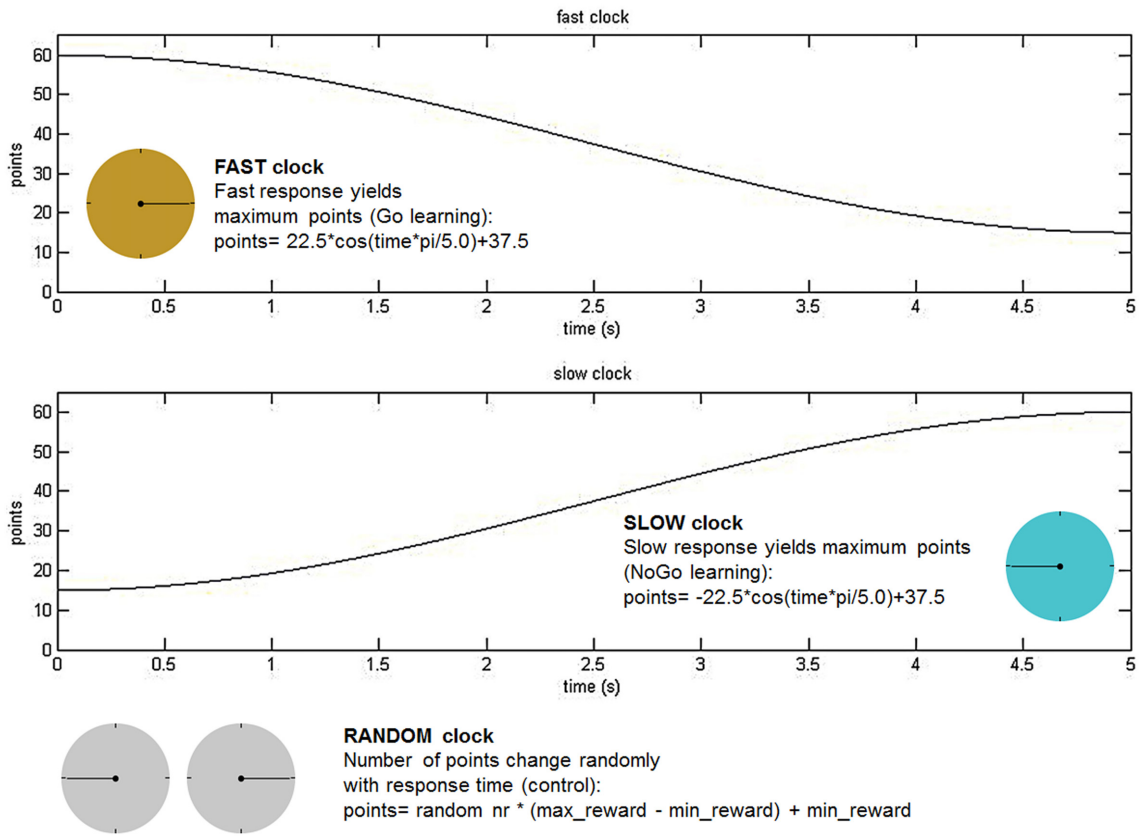

FIGURE 1 | Clock task. Reward values varied as a cosine function of response time. Subjects were instructed to figure out the optimal time point during a whole clock-arm turn of 5 s to stop the ticking clock and achieve maximum reward. In the fast clock condition, fast responses yielded the highest reward value (FAST clock for "Go learning"), whereas in the slow clock subjects had to wait longer to win maximum points (SLOW clock for "NoGo learning"). The RANDOM clock acted as a control condition, in which there existed no relation between response time and reward value. In all clock conditions random noise ranging between −5 and 4 points was added to the reward value in order to prevent subjects from memorizing an exact reward value at a specific response time. The three different clock types were presented in three separate runs for each clock consisting of 50 trials, which were counterbalanced across subjects and cycle phases.

further details regarding this task please also see Supplementary Figure S1.

BEHAVIORAL DATA ANALYSES

SPSS 19 was applied to analyze behavioral data. Prior to statistical analyses, mean RTs for each clock condition were calculated per subject. For that, RTs of less than 150 ms were discarded, since they were presumably caused unintendedly. Additionally, mean RTs of the first 12 trials (i.e., "first block") and the last 12 trials (i.e., "last block") of the three clock conditions were calculated for each subject (see Moustafa et al., 2008 for a similar procedure). Mean RTs of the first block allowed us to investigate the initial response speed indicating the subject's tendency to respond prior to learning in a state of uncertainty. Mean RTs of the last block indicated the optimized response speed near to the end of each clock condition, which represents a subject's best learning outcome. To obtain measures of Go and NoGo learning, relative response speed was determined. This was done by subtracting mean RTs of the FAST clock condition from mean RTs of the RANDOM condition (i.e., "relative speeding"), as well as subtracting mean RANDOM clock RTs from the mean SLOW clock RTs (i.e., "relative slowing"). This was also done for first and last block RTs, relative to first and last block RTs of the RANDOM clock, respectively. Since we were most interested in the time adjustment, i.e., the behavioral adaptation, over the course of each clock condition, differences between mean RTs of the last and the first block were calculated. Pearson correlations were performed to test for an association between RTs of the clock task and learning performance during the probabilistic learning task. A 2 × 2 repeated-measures ANOVA including the factors clock type (FAST, SLOW) and cycle phase (FP, LP) was computed to test for a possible effect on relative response time. Time adjustment and thus learning outcome, represented by the RT change from the first to the last block, was compared between the two cycle phases for both clock types using paired *t*-tests. Since we had clear a priori hypotheses regarding the direction of associations or differences in Go vs. NoGo learning in the two cycle phases, we report one-tailed significances if not otherwise indicated.

fMRI DATA ACQUISITION AND ANALYSES

The learning part of the probabilistic learning task, in which subjects were presented with positive or negative feedback according to their choices, was performed while brain activation was measured with fMRI. Imaging was conducted on a 3 T MRI scanner (Siemens TRIO). Thirty-three axial slices were acquired parallel to the anterior commissure – posterior commissure plane (voxel size = $2 \times 2 \times 2\,mm^3$, distance factor = 50%, descending direction) covering the whole brain. Functional images were obtained using a gradient echo planar imaging (EPI) sequence [repetition time (TR) = 2000 ms, echo time (TE) = 25 ms, field of view = 216 mm] resulting in a total of 738 image volumes. A high-resolution structural scan was acquired for each subject using a three-dimensional magnetization-prepared rapid-acquisition gradient echo sequence (MPRAGE) following the functional scans. Subjects viewed the experiment on the display through a mirror mounted on the head-coil and gave manual responses with a five-button keypad. Preprocessing and statistical analyses of functional data were performed using SPM8 (Wellcome Department of Cognitive Neurology, University College London, London, UK). Preprocessing steps included coregistration to the anatomical image, correction for motion artifacts (realignment and unwarping), for acquisition time differences (slice-timing), and for low-frequency fluctuations, normalization into standard stereotactic space (EPI template by the Montreal Neurological Institute, MNI), and spatial smoothing using a 6 mm full-width half-maximum isotropic Gaussian kernel. A general linear model (GLM) was defined to obtain parameter estimates of event-related activity in each voxel for each subject in both cycle phases. Positive and negative feedback (at time of feedback onset) were modeled as independent regressors convolved with the canonical hemodynamic response function (hrf). Linear t contrasts against implicit baseline were defined to explore the specific effects of each feedback type. The resulting contrast images were then passed to the second level analyses, in which correlations between feedback-related activity and RT adjustment in the clock task were assessed for both cycle phases using regression analyses.

All reported activations were significant at a voxel-level threshold of $p < 0.001$ uncorrected and survived a cluster-level family-wise error (FWE) correction for multiple comparisons with $p < 0.05$. For our a priori regions of interest, namely the RCZ and the VMPFC, we used small volume corrections (SVC, Worsley et al., 1996) and applied spherical volumes (radius = 10 mm) at the activation maxima reported by Klein et al. (2007b) for the RCZ (MNI-coordinates: $x = 6$, $y = 30$, $z = 29$) and by McClure et al. (2004) for the VMPFC (MNI-coordinates: $x = -8$, $y = 48$, $z = -4$). The coordinates by Klein et al. (2007b) were originally reported in Talairach space and thus converted to MNI reference space with GingerALE 2.1.1 using the Lancaster transformation (Lancaster et al., 2007). Activations corrected for small volume are reported at a voxel-level threshold of $p < 0.05$, corrected. Parameter estimates from the local activation maximum in the regions found to be associated with performance in the clock task were extracted with marsbar (available at: http://marsbar.sourceforge.net).

RESULTS

BEHAVIORAL RESULTS

First, we tested for coherence between time adjustment in the clock task and learning outcome in the probabilistic learning task. In line with our hypothesis, in the FP the initial response speed in the FAST clock condition (i.e., mean RT of the first block) showed a negative correlation with the frequency of choosing the best option (i.e., the letter "A") from new letter pairs in session 2 of the probabilistic learning task ($R = -0.509$, $p = 0.032$; **Figure 2A**). Hence, subjects who learned better via positive feedback were also responding faster at the beginning indicating that a preference for speeding up is also a form of "Go learning." RT adaptation in the FAST clock condition might be more intuitive for female subjects during the FP than during the LP, in which no such correlation could be observed ($R = 0.324$, $p = 0.129$). Also consistent with our predictions, during the FP the initial RT in the FAST clock was positively associated with the percentage of successful avoidance of the worst option (i.e., the letter "B") in new letter pairs ($R = 0.544$, $p = 0.022$; **Figure 2B**). According to this, a relative preference for avoidance learning was associated with a slower initial response tendency. Again, there was no equivalent correlation found for the LP ($R = -0.034$, $p = 0.454$). This further adds to the assumption that during the FP enhanced RTs in the FAST clock condition may be facilitated by a propensity for Go learning, while at the same time the ability to learn from punishment (NoGo learning) was compromised. Regarding the SLOW clock no correlations between initial response speed and performance in the learning task could be found. Also, there were no correlations between initial response speed and reinforcement learning during the LP. The observed associations in the FAST clock condition are in line with previous findings showing that the clock task is applicable to test for dopamine modulated learning performance in the sense of response time (Moustafa et al., 2008).

Next, we examined if the two learning processes, relative speeding (Go) and slowing (NoGo, for a similar procedure see Moustafa et al., 2008), were associated. As predicted, relative speeding and slowing were negatively correlated in both cycle phases (FP: $R = -0.918$, $p < 0.001$; LP: $R = -0.637$, $p = 0.007$; **Figure 3**). In the FP this association was slightly stronger than in the LP, which was indicated by the almost significant difference between the two correlations (Fisher's $z = -1.93$, $p = 0.053$, two-tailed). Subjects more prone to Go learning were good at speeding up during the FAST condition, while they had difficulties in slowing down (i.e., NoGo learning) in the SLOW condition. Again, this points to the functional opponency of the two learning processes and supports the view that the clock task is suitable to assess the cycle dependent modulation of temporal decision making.

Finally, we tested for an effect of menstrual cycle phase on RT adjustment and preferences for Go over NoGo learning and vice versa. Subjects showed a similar baseline response speed in both cycle phases with mean RTs in the RANDOM condition being equivalent in the FP and LP ($T = 1.035$, $df = 13$, $p = 0.320$, two-tailed; Mean RT RANDOM clock [mean ± sem]: FP = 2433 ± 199 ms, LP = 2146 ± 143 ms). To test for an effect of cycle phase on RT in the two clock types a 2 (clock type: FAST vs. SLOW) × 2

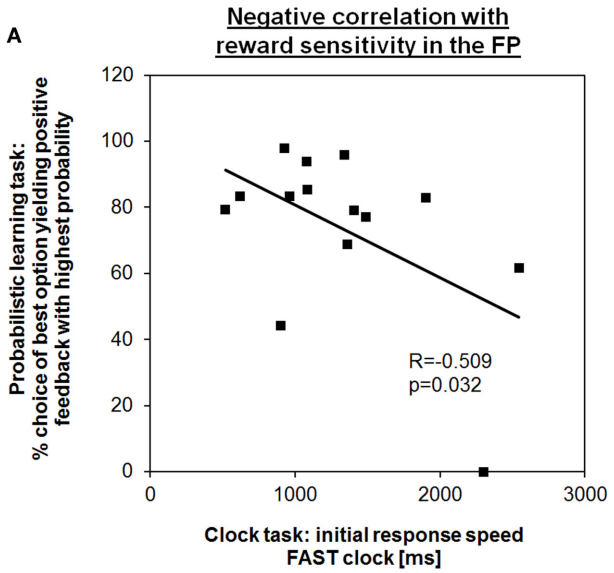

FIGURE 2 | Learning outcome from the probabilistic learning task predicts response speed in the first block of the fast clock condition during the FP. (A) Subjects who showed a higher percentage of choosing the best option in the second test session of the probabilistic learning task (i.e., "Go learners") were also better able to speed up during the fast condition at the very beginning. **(B)** In contrast, subjects who showed a higher tendency to avoid the most punished option (i.e., "NoGo learners") took longer to respond.

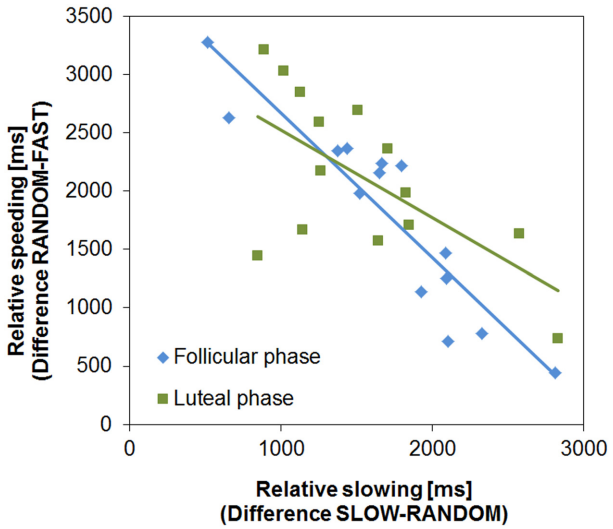

FIGURE 3 | Functional opponency of slowing and speeding. Relative slowing and speeding were negatively correlated in both cycle phases (FP: $R = -0.918$, $p < 0.001$; LP: $R = -0.637$, $p = 0.014$).

(cycle phase: FP vs. LP) repeated-measures ANOVA was run with the mean RTs. There was a main effect of clock type [$F_{(1,\ 13)} = 1119.01, p < 0.001$] in that RTs in the SLOW clock condition were generally higher than in the FAST clock ($T = 33.45$, $df = 13$, $p < 0.001$; Mean RTs [mean ± sem]: SLOW = 4243 ± 74 ms, FAST = 669 ± 54 ms,). However, there was no significant interaction between clock type and cycle phase in the different clock conditions for the RTs averaged over the complete run of 50 trials. The same applied to mean RTs of the last block of trials (i.e., optimized response speed). Solely, when considering the initial response speed during the first block of trials, a significant interaction between clock type and cycle phase could be found [$F_{(1,\ 13)} = 6.020, p = 0.029$]. *Post hoc t*-Tests revealed that in the SLOW clock condition subjects initially reacted more appropriately during the LP than during the FP ($T = -2.048$, $df = 13$, $p = 0.031$; initial RT SLOW clock [mean ± sem]: LP = 3574 ± 230 ms, FP = 3273 ± 190 ms).

The more exact measures of Go and NoGo learning in form of RT adjustment in relation to baseline RT in the RANDOM clock (i.e., relative speeding and relative slowing) revealed no interaction between cycle phase or clock type either. Nonetheless, the mean relative RTs from the last block of trials correspond numerically to the expected pattern (FAST condition [mean ± sem]: FP = 2183 ± 343 ms, LP = 1660 ± 251 ms; SLOW condition: FP = 1925 ± 356 ms, LP = 2456 ± 262 ms) although differences failed to reach statistical significance [clock type × cycle phase: $F_{(1,\ 13)} = 0.828$, $p = 0.379$]. Note that for the relative RTs a high value indicated better RT adjustment to the respective clock type.

Considering the course of learning by comparing time adjustment from the beginning to the end of the task (calculated by subtracting mean RT of the first 12 trials from those of the last 12 trials) revealed, however, a significant interaction between cycle phase and clock type [$F_{(1,\ 13)} = 5.707, p = 0.033$]. During the FP subjects displayed a higher level of response time adjustment in the SLOW clock condition by showing a greater RT change from the first to the last round than during the LP ($T = 2.412$, $df = 13$, $p = 0.016$; RT change [mean ± sem]: FP = 1304 ± 198 ms, LP = 939 ± 204 ms). In the FAST clock condition, mean RT changes in the two cycle phases did not significantly differ between cycle phases ($T = -1.435$, $df = 13$, $p = 0.088$; RT change [mean ± sem]: FP = −849 ± 152 ms, LP = −514 ± 152 ms). The fact that

the difference in RT change between the FP and LP was only significant in the SLOW clock condition task, but not in the FAST, suggests that slowing down puts more challenge on subjects during the FP than speeding up. Slow reponses might therefore require a higher level of adaptation due to the higher need for compensation during the FP. Hence, follicular subjects displayed an even greater RT change during the SLOW clock condition than during the FAST. **Figure 4** shows the RT changes of both cycle phases in the two clock conditions.

IMAGING RESULTS

To test for a link between response time adjustment in the clock paradigm and feedback-related activity in the probabilistic learning task, regression analyses with mean RTs of the different clock conditions and the neural responses based on the contrasts "negative feedback > baseline" and "positive feedback > baseline" were performed.

Follicular phase

We found several correlations between the optimized final response speed in the SLOW clock condition (i.e., mean RT of the last block) and feedback-related activity during probabilistic learning. Note that the SLOW clock condition presumably represented the hardest challenge for subjects during the FP. Regarding our a priori regions of interest, increased activation in the RCZ ($x = -8$, $y = 18$, $z = 40$) and reduced activity in the VMPFC ($x = 0, y = 42, z = -4$) in response to positive feedback as compared to baseline were predictive of slower RTs in the last block of the SLOW clock condition. Furthermore, signal increases in the left IFJ ($x = -34, y = 4, z = 38$) in response to positive feedback were positively associated with RTs in the SLOW clock condition (see **Figure 5A** for clusters found to be associated with effective RT adaptation based on the contrast "positive feedback > baseline").

Interestingly, the same neural correlates of optimal RT adaptation in the SLOW clock were found in the "negative feedback > baseline" contrast. A slower optimized response speed could be predicted by a signal increase in the RCZ ($x = -10$, $y = 18$, $z = 40$), whereas a poorer RT adaptation toward the end of the SLOW clock condition was accompanied by increased activation in the VMPFC ($x = 0$, $y = 48$, $z = 2$). In addition, stronger responses in the left IFJ ($x = -42$, $y = 0$, $z = 34$) were predictive of slower final RTs (**Figure 5B**). Furthermore, during both feedback types optimized RTs in the SLOW clock correlated positively with activations in the precentral sulcus and the inferior temporal lobe and negatively with activation in the posterior superior temporal sulcus (post STS) and the middle occipital cortex (MOC) (**Table 1** lists all activations that were found to be associated with optimized response speed in the SLOW clock condition during the FP). The correlations between optimized RT in the SLOW clock condition and the parameter estimates extracted from the local activation maxima of the clusters in the RCZ, IFJ, and VMPFC further point to the resemblance of the brain-behavior correlations in response to positive and negative feedback and indicate that these results were not driven by outliers (**Figure 6**). No correlations were found between feedback-related brain activity and RTs in the FAST clock condition.

Luteal phase

Feedback-related activity in the LP was not associated with RT adaptation in any of the three clock types. This corresponds to the lack of correlations between RT adaptation and the behavioral data of the probabilistic learning task in the LP.

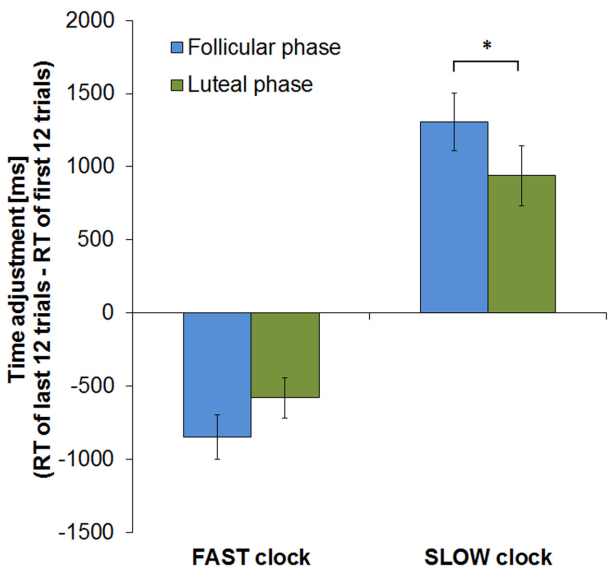

FIGURE 4 | RT change from first to last block of the clock task in both cycle phases. In both clock conditions subjects showed greater RT change in the course of the experimental run during the FP (*$p < 0.05$). Error bars represent standard error of the mean (sem).

DISCUSSION

In this study we investigated whether naturally occurring variations in E2 and PROG levels influence the ability to adapt response speed in the sense of Go vs. NoGo learning (i.e., speeding up vs. slowing down) over the course of the menstrual cycle. Indeed, we observed that in the FP as compared to the LP subjects displayed a higher need for adaptation of their response speed in the condition that required slower responses for reward maximization. The rather compromised ability to adapt to a slow response speed during the FP was also reflected in the observed correlations between optimized response speed in the last block of the SLOW clock condition and follicular brain activation in response to positive and negative feedback during a probabilistic learning task. While increased activation of the RCZ and the IFJ was associated with better RT adaptation to a slow speed, strong responses in the VMPFC predicted poorer performance in this condition.

In sum, our results indicate an effect of menstrual cycle phase on NoGo learning processes in the temporal domain. These findings support our hypothesis that increased E2 levels during the FP may lead to a Go learning bias and an impaired ability to learn via the NoGo pathway. Against the background of growing evidence from rodents that describes an opposing effect of E2 and PROG on striatal DA levels, in that E2 enhances whilst PROG

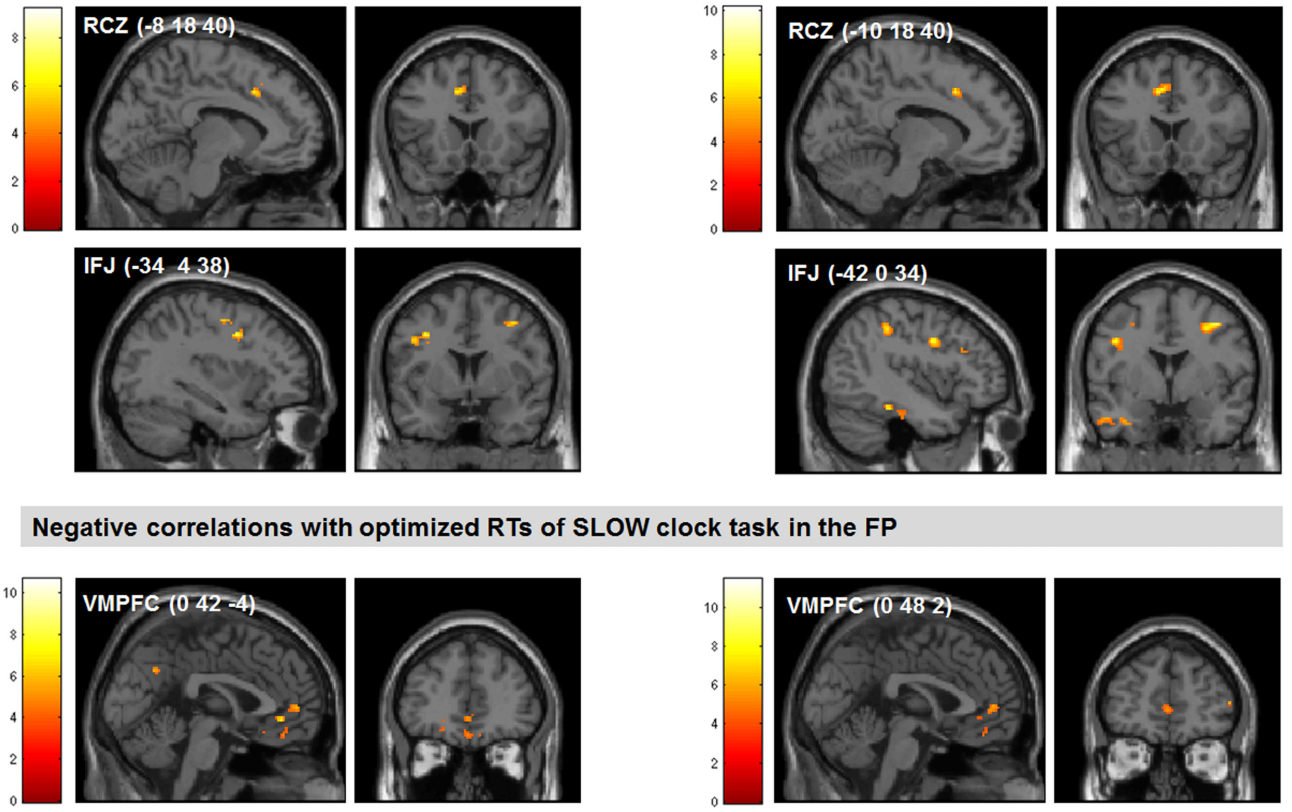

FIGURE 5 | Feedback-related activity during probabilistic learning predicted performance in the last block of the SLOW clock during the FP. (A) Correlations between neural responses based on the contrast positive feedback > baseline in the probabilistic learning task and optimized RTs of the last block during the SLOW clock condition. **(B)** Correlations between neural responses based on the contrast negative feedback > baseline in the probabilistic learning task and optimized RTs of the last block during the SLOW clock condition. Displayed positive correlations survived FWE correction for multiple comparisons at cluster-level ($p < 0.05$). The negative correlations are reported at $p < 0.05$, SVC (based on the coordinates reported in McClure et al., 2004).

acts inhibiting on dopaminergic tone (Luine and Rhodes, 1983; Dluzen and Ramirez, 1984; Majewska et al., 1986; Mauvais-Jarvis et al., 1986; Lévesque et al., 1989; Becker, 1999), our data are consistent with previous studies reporting an effect of striatal DA on action selection (Frank et al., 2004; Klein et al., 2007b; Moustafa et al., 2008). All these previous results on the association between DA and action selection had been derived from testing individuals with differences in dopaminergic functioning (i.e., Parkinson patients on and off medication or carriers of specific genetic polymorphisms concerning D2 receptor density). Collectively, they point to an increased preference for Go over NoGo learning in a state of high as compared to low dopaminergic tone. This is in line with our observation of an impaired learning ability in the SLOW condition during the FP. In that phase high levels of E2 are unopposed by PROG and are presumed to increase DA levels, which is why they should indirectly cause a Go bias. In the present study, subjects in the FP displayed a higher need for RT adaptation in the SLOW clock condition, which acted as a measure of NoGo learning performance. Considering the greater change of RT over the course of the SLOW clock during the FP, our results suggest that subjects were able to compensate for their impaired ability to adapt to a slow pace, by taking more effort in response speed adaptation. However, this adaptation may not have been as sufficient as the performance in the LP, which was indicated by slightly poorer relative slowing of follicular subjects. The fact that relative speeding and slowing did not significantly differ between the two cycle phases potentially indicates that effects of naturally occurring hormonal shifts during the menstrual cycle are not necessarily very strong. As a result, they may rather be compensated without greater effort in an explicit behavioral task such as the clock task. Altogether, our data support the theoretical model of a DA dependent learning style (e.g., Frank et al., 2004; Cools, 2008) and propose natural variations of the steroid hormones E2 and PROG as other important factors that need to be considered. Apart from its biasing effect toward the Go pathway in reward-based learning, E2 has also been found to increase the internal

Table 1 | Brain activations in response to negative and positive feedback during probabilistic learning which correlated with optimized RTs (last block) of the SLOW clock condition in the follicular phase.

Region	Neural activations in response to positive feedback (positive feedback > baseline contrast) associated with last block RTs of the SLOW clock condition					Neural activations in response to negative feedback (negative feedback > baseline contrast) associated with last block RTs of the SLOW clock condition				
	x	*y*	*z*	*T*	Cluster size	*x*	*y*	*z*	*T*	Cluster size
POSITIVE CORRELATIONS										
RCZ	−8	18	40	6.44	77	−10	18	40	7.20	126
L IFJ	−34	4	38	7.29	48	−42	0	34	7.47	99
R SFS/precentral sulcus	38	0	48	6.06[b]	68	38	0	48	8.15	112
L Inferior temporal lobe	−44	−36	−18	9.24	142	−44	−36	−18	10.15	208
NEGATIVE CORRELATIONS										
VMPFC	0	42	−4	5.041[a]	4	0	48	2	5.36[a]	13
R Posterior STS/MTG	48	−52	24	10.65	177	52	−52	18	11.45	139
MOC	−44	−76	34	8.23	116	−42	−76	34	5.45	92

Activations are reported at $p_{FWE} < 0.05$ (cluster-level) if not otherwise indicated.

[a] *p<0.05, SVC.*

[b] *p<0.001, uncorrected (voxel-level).*

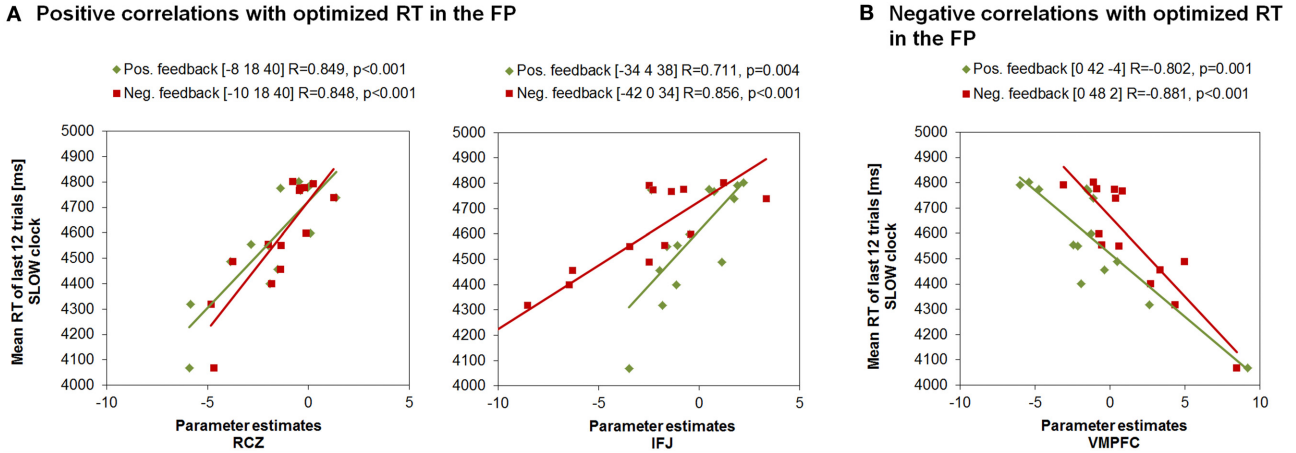

FIGURE 6 | Correlations between RTs of the last block in the SLOW clock and the parameter estimates of peak voxels in feedback-related clusters found to be associated with response time adjustment in the SLOW clock during FP. (A) Increased activation in RCZ and IFJ predicted effective response time adaption in the SLOW clock. **(B)** Increased activation in the VMPFC was associated with compromised RT adaptation in the SLOW clock.

clock speed (Sandstrom, 2007; Pleil et al., 2011). In our study, subjects in the FP might therefore have overestimated the passing time and given their response relatively sooner. This explanation could also account for the initially faster and thus less suitable response speed in the SLOW clock condition that was observed during the FP. Again, this accelerating effect of E2 is presumably driven by an interaction with the DA system. DA has been found to be involved in time perception by studies reporting an increase of the internal clock speed in response to DA agonists (e.g., Meck et al., 1996). Hence, in the presence of high E2 levels one may speculate that heightened DA levels might have also promoted an acceleration of internal clock speed. This assumption also seems plausible when considering that both enhanced DA levels as well as heightened E2 may increase individual delay discounting tendencies in man and animals (Winstanley, 2011; Smith et al., 2014), which in part depend on the subjective feeling of passing time as well.

As to the translation of adjusting response time to a fast vs. a slow speed to Go vs. NoGo learning, we argue that these two learning styles, which are usually assessed by reward-based learning tasks (i.e., reinforcement learning), are also involved in response time adjustment. The fact that we applied both types of tasks, the clock task for time adjustment and the probabilistic learning task for reward-based learning, allowed us to examine if performance in the two paradigms both rely on Go and NoGo learning processes and how the two tasks are associated with each other. We found correlations between the initial response speed in the FAST clock condition and the learning outcome in the probabilistic learning task during the FP that support the view that RT adjustment to a fast vs. a slow speed is equivalent to Go vs. NoGo

learning processes (Moustafa et al., 2008). Specifically, subjects who were better in speeding up also showed higher reward sensitivity in the probabilistic learning task, whereas their avoidance of the punished option was less successful. Thus, the Go learning condition of the clock task (i.e., the FAST clock) was positively associated with Go learning as well as negatively associated with NoGo learning in feedback learning.

On the neural level our results indicate antagonistically acting brain-behavior correlations. We found that in the FP increased activity in the RCZ was predictive of a successful RT adaptation toward the end of the SLOW clock condition. Since the RCZ plays an important role in NoGo learning, as indicated by its frequent observation during learning from errors and negative feedback (Fiehler et al., 2004; Klein et al., 2007a; Jocham et al., 2009), an increased activity in this region could have helped subjects to perform better in a task that required NoGo learning in the form of waiting longer for a higher reward. Klein et al. (2007b) applied a reinforcement learning task resembling our version of the probabilistic learning task and compared two groups differing in their genetically determined D2 receptor density. The group with a high receptor density showed an increased signal in the RCZ in response to negative feedback and this group was also better at avoiding a negative outcome in the behavioral post-test. The D2 receptor type has been proposed to be implemented in the NoGo pathway (Frank et al., 2004) and therefore an increased receptor density of this type should cause a bias toward NoGo learning. Since the high E2 levels are thought to downregulate D2 receptor density and may decrease receptor binding (Bazzett and Becker, 1994; Becker, 1999), individuals that show higher sensitivity of the RCZ to NoGo signals in form of negative feedback might be better adapted to succeed in the SLOW condition of the clock task. The cluster in the RCZ found in this study did not survive small volume correction based on the previously published coordinates by Klein et al. (2007b), which is why our result considering the RCZ may be interpreted cautiously. It might be that small location differences between the RCZ cluster reported here and that of Klein et al. (2007b) are due to fact that we only tested female subjects while the other study investigated men. In fact, sex differences in brain volume (e.g., Cosgrove et al., 2007) may account for slight locational drifts to some extent. Nonetheless, the anatomical location of the cluster reported here corresponds to the literature about the RCZ (see for example Ridderinkhof et al., 2004). Thus, our results further underline the importance of the RCZ for NoGo learning by pointing out that in the FP subjects may have benefited from a stronger RCZ response during feedback learning, which also led to an advantage in the SLOW clock condition.

Similarly, in the FP subjects with heightened activity in the IFJ during feedback processing were more successful to adapt in the SLOW clock. Since the IFJ has been consistently implicated in cognitive control (Brass et al., 2005; Derrfuss et al., 2005), increased activity in this region might have helped subjects in the FP to perform better in this counterintuitive task, in which they had to counteract the tendency for immediate responding. In contrast, decreased activation in the VMPFC was associated with better RT adaption to a slow response speed. This is in line with observations that increased activation in this brain region is associated with preferences for immediate reward and may promote delay discounting tendencies both in the context of longer time scales (McClure et al., 2004) as well as in the range of seconds (McClure et al., 2007). In addition, the VMPFC may also play a role in the representation of relative reward value und reward-related preferences (e.g., Grabenhorst and Rolls, 2011). In the context of the probabilistic learning task this region has been found to show an increased activity in the post-learning test phase in response to Go trials, in which subjects should select the most rewarded option (i.e., "AC," "AD," "AE," "AF") (Jocham et al., 2011). More importantly, this effect was found in subjects having received a dose of amisulpride, a D2 antagonist, but it was absent when the same subjects received placebo. Considering this, the VMPFC might be especially implicated in value-based decision making that involves Go learning processes. Therefore, a heightened activity in this region during feedback processing may potentially explain subjects' poor performance in the NoGo condition of the clock task, since a strong response in this brain region could be interfering with a task that requires the NoGo pathway.

In sum, the brain-behavior correlations suggest that effective RT adaption to a slow speed during the FP may be related to enhanced top-down processing involving increased activation in regions implemented in cognitive control and monitoring of behavioral outcomes. On the other hand, enhanced bottom-up processing in areas implicated in reward valuation might be associated with compromised subsequent RT adaptation. The fact that these correlations were only found in the last block of the SLOW clock condition may reflect a subject's maximum individual capacity to compensate for her compromised ability to be patient and to finally withhold the initial urge to respond rather rapidly during the FP. Correspondingly, we found no consistent correlations during the LP in the absence of this need for compensation. The FAST clock condition in turn should also not require much compensation in subjects during the FP, which might explain the lack of equivalent correlations between RT adaptation and brain activity during feedback learning in this condition. This absence of neural correlates predicting the ability to speed up response speed in the Go condition of the clock task corresponds well to the findings of Klein et al. (2007b) who reported mainly differential activation associated with negative feedback (i.e., NoGo learning) when comparing subjects with different D2 receptor densities. Interestingly, in the present study effective RT adaptation could be predicted by neural responses from the same regions independent of feedback-type (see **Table 1**). It might be that the successful compensation for a compromised capacity for NoGo learning in the FP required a general increase of performance monitoring resulting in an enhanced recruitment of RCZ and IFJ during feedback-processing *per se*.

Important to note, our results indicate neural correlates predicting the ability to adapt response speed on the interindividual level. Although we can only speculate that this may have been caused by possible interactions with other factors we did not include in our design. First, the time of the day on which the tests were carried out could not be controlled across participants. There is, however, some evidence that points to an interaction between time perception and circadian rhythm (e.g.,

Lustig and Meck, 2001; Kuriyama et al., 2003; Bussi et al., 2014). Also we did not account for possible effects by other hormones. The steroid hormone cortisol has been shown to influence delay discounting (Takahashi, 2004) and might therefore also play a role in time adjustment. Furthermore, genetically determined differences in dopaminergic functioning could have affected our results. For instance, Jacobs and D'Esposito (2011) tested working memory performance of women during different phases of their menstrual cycle and found an interaction between the cycle phase and a genetic polymorphism in the catechol-O-methyltransferase (COMT) gene, the Val^{158}Met polymorphism. This polymorphism determines the activity of the COMT enzyme in that carriers of the *met/met* allelic variant have decreased enzymatic activity in the prefrontal cortex and therefore higher DA levels as compared to individuals with the *val/val* genotype (e.g., Cai and Arnsten, 1997; Weinberger et al., 2001; Gibbs and D'Esposito, 2005). In their study Jacobs and D'Esposito (2011) found that *val/val* subjects showed increased performance during the FP when E2 reaches peak level, while subjects with the *met/met* genotype performed better during menses when E2 is low. Since DA function follows an inverted-U-shaped curve with deficient or excessive DA levels leading to less optimal DA functioning, increases of E2 during the menstrual cycle might evoke different effects depending on the "baseline" DA function of an individual (for an overview see also Colzato and Hommel, 2014). This interaction between the COMT Val^{158}Met polymorphism and the effect of E2 on DA related cognitive processes has recently also been observed in the context of delay discounting (Smith et al., 2014). In this study subjects showed a reduced bias for sooner/smaller rewards in the FP compared to menses, an effect mainly driven by carriers of the *val/val* genotype.

Taken together, our findings provide initial evidence for an effect of menstrual cycle phase on the preference for Go over NoGo learning in a response time adjustment paradigm. During the FP in the presence of high E2 levels and thus presumably elevated DA levels, NoGo learning was impaired and therefore the ability to slow down and to wait patiently was impeded. During this cycle phase, effective adaptation to slow speed might have been achieved by (1) increased performance monitoring during feedback processing in the RCZ and IFJ and (2) decreased reward-related responses of the VMPFC in order to suppress the initial urge to respond rapidly and to counteract the phase-specific Go bias. The fact that we found no corresponding brain-behavior correlations during the LP emphasizes the absence of the need for compensation in the presence of high PROG levels that should act positively on the NoGo pathway. In summary, these data suggest a cycle dependent modulation of temporal decision making requiring Go and NoGo learning systems.

Our results also add further evidence to the more and more described neuroregulatory effects of E2 and PROG on dopamine-related behaviors. A growing number of studies focused on endogenous variations of E2 and PROG during the menstrual cycle and reported cycle-dependent brain activation, for instance enhanced reward processing during the FP (Caldú and Dreher, 2007; Dreher et al., 2007). Moreover, cycle phase has been shown to affect working memory (Gasbarri et al., 2008; Jacobs and D'Esposito, 2011) and inhibitory control (Colzato et al., 2010). Neuroendocrinological research has only recently begun to investigate naturally occurring differences in dopaminergic transmission by taking into account hormonal shifts during the menstrual cycle. Hence, our study contributes important insights into the linkage between estradiol induced dopamine increases and their impact on temporal decision making and response time adaptation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2014.00401/abstract

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The impact of sex hormone concentrations on decision-making in females and males

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Human decision-making has been frequently studied and sex differences have been reported. Interestingly, previous results of hormone concentration on decision-making are somewhat inconsistent, regarding the impact of menstrual cycle phase in women or the influence of testosterone concentration on decision-making in women and men. However, the influence of the female sex hormone concentration (estradiol, progesterone) and the impact of oral contraceptive intake have rarely been examined and data regarding the effect of daytime variations of male testosterone are lacking. Moreover if personality factors such as sensation seeking, impulsivity, and anxiety influence decision-making, sex-specific effects, act as modulators is unclear. In the present study 71 women and 45 men were enrolled. All participants performed an evaluated decision-making task measuring risk-taking behavior on the basis of contingencies (Haegler et al., 2010), which can be carried out several times without a learning effect. Saliva samples were collected to obtain estradiol, progesterone, and testosterone levels. Additionally, all participants completed questionnaires measuring various personality factors. Data analysis revealed no sex differences in decision-making and no significant impact of testosterone concentration on behavioral performance in women or men. However, a significant negative correlation between progesterone concentration of women in the luteal phase and their performance in the risk-averse condition was obtained. Interestingly, a significant correlation between trait anxiety and decision-making occurred in females and males. Despite similar risky decision-making of women and men and no influence of testosterone concentration, menstrual cycle phase showed an effect on risk taking in women. In contrary to other studies, our findings provide rather subtle evidence for hormonal influences in decision-making, which may be primarily explained by task factors.

Keywords: sex, decision-making, testosterone, progesterone, estradiol, risk-taking

INTRODUCTION

Every day is characterized by lots of decisions that we make, covering basic needs such as what to eat and drink and higher-order motives, e.g., who will I talk to during lunch break. In general, decision-making plays a pivotal role in our lives and comprises a complex process of assessing and weighing short-term and long-term costs and benefits of competing actions (van den Bos et al., 2012). The output of the decision-making process, i.e., which action is to be taken, is determined by an interaction between impulsive or emotionally based systems, responding to immediate (potential) rewards as well as losses or threats, and reflective or cognitive control systems controlling long-term perspective (Bechara, 2005).

One important factor of decision-making is risk taking, meaning the tendency of preferring an action with a possible large profitable or aversive outcome, although unlikely, over an alternative action with small profitable more likely outcome. Previous research in this regard has mostly demonstrated that women show less risk taking behavior than men in various domains (e.g., Jianakoplos and Bernasek, 1998; Byrnes et al., 1999; Zuckerman and Kuhlman, 2000; Zuckerman, 2006).

As pointed out by Stanton et al. (2011), economic risk is a domain that most individuals are frequently confronted with and thus of particular interest. One approach to measure an individual's propensity for risk taking in the face of monetary rewards and punishment is the Iowa Gambling Task (IGT, Bechara et al., 1994; Bechara, 2005). In this rather "economic" decision-making task participants learn to differentiate long-term advantageous from long-term disadvantageous decks of cards through exploration. Here, it is well-established that men and women differ in decision-making performance, with men choosing more cards from the long-term advantageous decks than women within the standard number of 100 trials (Bolla et al., 2004; Overman et al., 2006; Visser de et al., 2010). According to a recent review on

sex differences in IGT performance by van den Bos et al. (2013), sex differences only emerge after about 60 trials, meaning that in the very beginning, females and males perform similar. Later on males seem to shift earlier to applying the correct rule by taking more cards from the long-term advantageous decks, while women need more time. In the end, both sexes prefer the long-term advantageous decks, however, women need longer before doing so consistently. As female reward sensitivity and processing are shaped by the menstrual cycle this could be related to the obtained sex difference, however, previous attempts to investigate this factor did not show a clear effect (Reavis and Overman, 2001; van den Bos et al., 2007).

Another crucial aspect might be testosterone concentration, which has been frequently linked particularly to risky decision-making. Recent data from Stanton et al. (2011) indicate that females and males with high testosterone levels show more risky behavior than those with low testosterone concentration, with a more pronounced effect in women. Besides age effects (e.g., Diver et al., 2003), it has been argued that testosterone concentration fluctuates across the day, with higher values after awakening than in the afternoon or evening in males (Axelsson et al., 2005). Until now it is unclear whether this diurnal variation influences decision-making and particularly risk taking.

Moreover, other studies in humans employing decision-making paradigms such as the Game of Dice Task (Starcke et al., 2008) and the Balloon Analog Risk Task (Lighthall et al., 2009, 2011) have not observed differences between men and women regarding risk-based decision-making. Furthermore, in the Cambridge Gambling Task men and women did not differ in risk-taking or impulsivity, but only in risk-adjustment, i.e., adjusting betting behavior according to the likelihood of winning (Deakin et al., 2004; van den Bos et al., 2014).

At present therefore it is not exactly clear under which task conditions men and women differ in decision-making and how this relates to differences in sex hormone concentration due to menstrual cycle phase (progesterone, estradiol) or daytime (testosterone). Such knowledge however will give more insight in how and under which circumstances sex differences in decision-making can be observed.

The aim of the present study therefore was to investigate the impact of (a) menstrual-cycle phase vs. oral contraceptive intake, (b) diurnal variation of testosterone in males and (c) testosterone concentration in females and males on decision-making. Besides group differences, we also analyzed potential associations between behavioral performance, hormonal parameters, and self-report questionnaire date.

METHODS AND MATERIALS

SAMPLE

Seventy-one right-handed healthy females aged 19–37 years (mean age 23.8 years, $SD = 3.7$) participated in the study. When contacted, female participants were asked whether they were taking oral contraceptives and if not, were asked to report their menstrual cycle phase and cycle duration. Based on this information they were assigned a testing date. Only females who reported regular cycle duration (range: 25–35 days, mean days = 28.3, $SD = 2.5$) were included. At the day of testing, 22 females were in their follicular phase (days 1–12 of menstrual cycle; FO; mean age 23.6 years, $SD = 3.8$), 26 were in their mid-luteal phase (days 18–25 of menstrual cycle; LU, mean age 24.3 years, $SD = 3.8$) and 23 were taking oral contraceptives (OC, mean age 23.3 years, $SD = 3.5$). All females were tested between 9 and 11 a.m.

Moreover, 45 right-handed males aged 20–36 years (mean age 24.8 years, $SD = 3.1$) were tested. Twenty-two were tested before noon (9 to 11 a.m.) when testosterone levels are supposed to be higher (mean age 24.4 years, $SD = 2.0$), while the other 23 were measured in the late afternoon between 5 and 7 p.m. (mean age 25.1 years, $SD = 3.8$).

Groups did not differ in age [$F_{(4,\ 111)} = 1.001$, $p = 0.410$], or educational level [$F_{(4,\ 111)} = 1.148$, $p = 0.338$].

Additionally, all participants were asked to fill out several questionnaires tapping verbal intelligence (Mehrfachwahl-Wortschatz-Intelligenztest Version B, MWT-B, Lehrl, 2005), sensation seeking (SSS-V, Zuckerman, 1994), impulsivity (Barrett Impulsivity Scale, German Version: Preuss et al., 2008), depression (Beck Depression Inventory II BDI, Beck et al., 2006) and anxiety (State trait anxiety inventory, STAI, Laux et al., 1981).

Participants were recruited by advertisements at the University of Vienna and the Medical University of Vienna, Austria. All participants were screened for history of any psychiatric or mental disorder by using the German version of the structured interview of DSM IV (SCID; Wittchen et al., 1997). Written informed consent was obtained from all subjects prior to the examination and the study was approved by the local institutional review board.

SALIVA SAMPLES

To obtain actual estradiol, progesterone and testosterone levels saliva samples were collected on the day of testing. Saliva samples have been shown to have great potential for studying ovarian and androgen hormone levels as a reliable, feasible, and non-invasive method (e.g., Gandara et al., 2007). Before we started obtaining saliva samples we asked participants to wash out their mouth with water. In order to avoid arbitrary results we collected saliva samples for each hormone every half hour, thus we collected three samples per hormone in total (multiple sampling). Participants were instructed to fill a small plastic vial with at least 1.5 ml saliva (max. 3 ml) using a straw to stimulate saliva flow. Participants' collection vials were sealed after each collection and frozen immediately in accordance with previous research on sample storage (see Gröschl, 2008).

Saliva samples were analyzed by the European Institute for Salivary Analysis (Swiss Health Med, Aying, Germany) using an enzyme-linked immunoassay method from DRG (DRG Marburg, Germany; Salivary Estradiol ELISA SLV-4188, DRG Salivary Progesterone ELISA SLV-2931, DRG Salivary Testosterone ELISA SLV-3013). Analytical sensitivity (confidence interval 95%) was 0.4 pg/mL (Estradiol), 3.9 pg/mL (Progesterone), and 1.9 pg/mL (Testosterone). For estradiol, intra- and inter-assay coefficients were 3.8 and 2.6%, respectively. For Progesterone, intra- and interassay coefficients were 7.7 and 5.3%, respectively. For testosterone, intra-assay coefficients were <4% and inter-assay CV < 5%.

For details on hormone concentration of groups see **Table 1**.

Table 1 | Description of groups including sociodemographic, hormonal, and neuropsychological means (standard deviations in parentheses) and *p*-values.

Females	Follicular ($n = 22$)	Luteal ($n = 26$)	Oral contraceptives ($n = 23$)	*p*-values
Age	23.6 (3.8)	24.3 (3.8)	23.3 (3.5)	0.610
Estradiol (pg/mL)	3.9 (1.3)	5.6 (7.2)	3.9 (1.4)	0.351
Progesterone (pg/mL)	94.4 (113.1)	197.1 (133.3)	65.6 (21.7)	**<0.001**
MWT-B (raw score)	28.1 (3.4)	28.4 (3.2)	28.7 (3.0)	0.932
TMT-A (raw score)	19.5 (3.8)	20.2 (6.9)	20.1 (4.8)	0.886
TMT-B (raw score)	36.1 (10.0)	36.6 (13.0)	35.6 (10.1)	0.949
Males	**Morning ($n = 22$)**	**Afternoon ($n = 23$)**	***p*-values**	
Age	24.4 (2.0)	25.1 (3.8)	0.409	
Testosterone (pg/mL)	70.0 (20.7)	56.6 (17.6)	**0.023**	
MWT-B (raw score)	27.3 (2.5)	28.8 (2.4)	**0.047**	
TMT-A (sec)	18.6 (6.6)	19.9 (5.9)	0.483	
TMT-B (sec)	36.0 (13.5)	34.7 (9.3)	0.710	
Testosterone	**Females HT ($n = 36$)**	**Females LT ($n = 35$)**	**Males HT ($n = 22$)**	**Males LT ($n = 23$)**
Age	23.7 (4.0)	23.8 (3.3)	24.3 (2.2)	25.2 (3.7)
Testosterone (pg/mL)	12.9 (11.7)	2.7 (1.5)	79.9 (12.8)	47.2 (10.5)

Significant p-values are marked in bold.

Note: MWT-B, Mehrfachwortwahltest-B measures verbal intelligence; TMT-A/-B, Trail Making Test -A/-B measure executive functions. HT, high testosterone concentration; LT, low testosterone concentration.

DECISION-MAKING TASK

For this study we chose out of a battery of decision-making tasks which can be performed repeatedly without learning effect. Such tasks include for example the Balloon Analog Risk Task (Lejuez et al., 2002) the Cambridge cognition task (http://www.cambridgecognition.com/), the Game of Dice Task (Brand et al., 2005), and the Haegler's Risk Game (HRG). The HRG is based on a card game which is described in great detail elsewhere (Haegler et al., 2010). Briefly, participants were told that they would see an unknown amount of play card pairs with values from 1 to 10, 1 being the smallest and 10 being the highest possible card. After seeing the first card, participants had to decide whether the second card, would be either higher or lower than the first card. If their choice was correct, participants gained reward points. If their choice was wrong, participants lost points.

Starting with 0 points, reward points were accumulated over the rounds, while it was also possible to accumulate a negative amount of points. Participants were instructed that reward points were valuable, and it was the goal of the game to accumulate as many points as possible. They were paid a fixed amount of money, which they were aware of before the study started, but there was no mapping between points and monetary reward. Nevertheless, participants were instructed to play the HRG with the objective of winning as many points as they could. In total, 100 card pairs were presented per game cycle, taking approximately 5 min for completion. The first card was pseudo-randomized and ranged from 2 to 9. The second card was selected by chance ranging from 1 to 10 but always occupying a different value than the first card. Presentation of the first card was accompanied by additional information: the amount of points to be won if the participants' choice was correct was shown in green ink; the amount of points that could be lost was shown in red ink. Additionally, a green–red bar indicated the ratio between the possible number of points to be won or lost. Participants indicated their choice by either pressing the lower or the higher button in the response panel. After making their choice the points were either added or subtracted from the total amount of points depending on the accuracy of the response. Additionally, the second card appeared highlighted by a green or red box in combination with a dialog window saying either "You win!" or "You loose!" depending on the accuracy.

Since the second card was drawn completely random, the statistical probability for the second card to be lower varied according to the value of the first card. As an example, if the first card carried the value 2, the probability for the second card to be lower was 1/9. If the first card carried the value 9, the probability for the second card to be lower was 8/9. The amount of points to be won or lost for a correct or incorrect choice varied and was directly correlated to the statistical likelihood of the event to occur. The probability of the second card to be higher if the first card carried a value $x \in \{2, \ldots, 9\}$ was $p_{\text{higher}} = (10 - x)/9$, therefore, the points that could be lost were $(10 - x) \times 10$ and the points that could be gained were $90 - [(10 - x) \times 10]$. For the second card to be lower, the probability was $p_{\text{lower}} = 1 - p_{\text{higher}}$, resulting in either a deficit of $90 - [(10 - x) \times 10]$ points or a debit of $(10 - x) \times 10$ points.

Due to the fact that the points to be won or lost were opposed to the probabilities, the chances of winning or loosing were random, resulting on average in a total amount of 0 points at the end of the game cycle. Hence, no strategy could be learned which would help the participants to win the game. Thus, in contrast to other gambling games like for instance the IGT, participants can play the HRG multiple times without a learning effect.

Participants were considered as playing more risky if they chose higher while the first card was 6, 7, 8, or 9 or if they chose lower while the first card was 2, 3, 4, or 5 more often. The key dependent variable was, therefore, the summed number of risky selections of each participant. Accordingly, the pairs 2-lower and 9-higher, 3-lower and 8-higher, 4-lower, and 7-higher, as well as 5-lower and 6-higher were combined by summing up the number of single selections, due to equal probabilities. This resulted in a total of 4 risk values per participant. On average each card value of the first card appeared 12.5 times during a game cycle, hence, the average number of presentations of one card pair was 25 per game cycle. During each game cycle the response time,

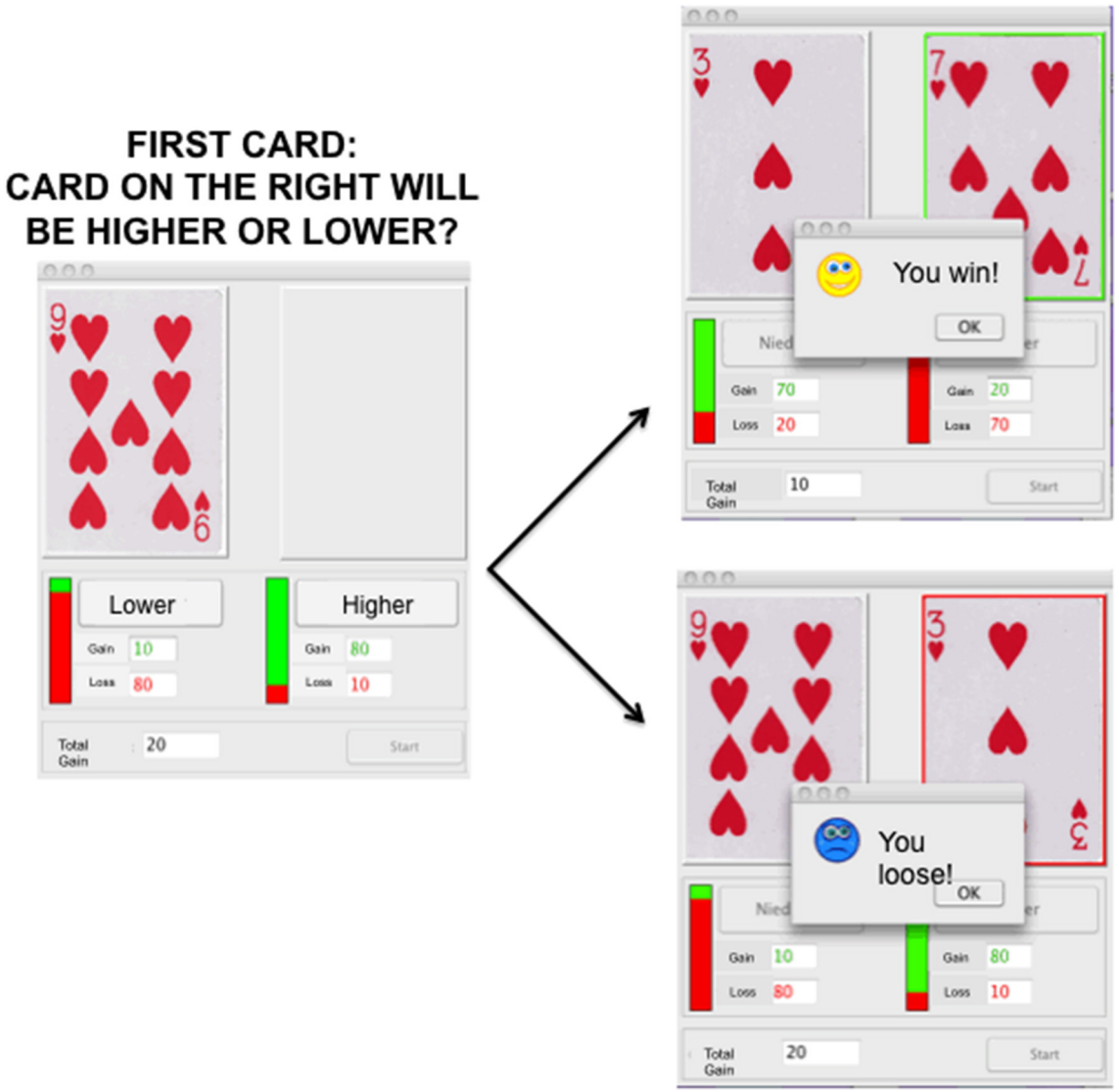

FIGURE 1 | Illustration of Haeglers Risk Game depicting the screen with one card on the left and the option of the participant to choose a lower or higher card will be displayed on the left. On the right the two alternatives, depicting either a win (top), or a loose (bottom) trial, are illustrated.

meaning the time from the display of the first card until participants pressed either the higher or the lower button, as well as each choice made by the participants were monitored. Please see **Figure 1** for illustration of the task.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS 20.0 and level of significance was set at $p = 0.05$. We performed three different analyses in order to compare the three female groups (FO vs. LU vs. OC), the two male groups (morning vs. afternoon) and—in line with the paper by Stanton et al. (2011)—the impact of testosterone level (high vs. low concentration in females and males).

Number of card selections and reaction times in the HRG were analyzed using mixed-model ANOVAs with risk selection as within-subject factor and group as between-subject factor. For significant effects partial-eta squares are listed as estimates of effect size. In cases of violations of sphericity, statistical tests involving the risk selection factor employed Greenhouse-Geisser correction. All *post-hoc* results were Bonferroni corrected.

Group differences regarding neuropsychological parameters (MWT-B, TMT) and the questionnaire data (BDI, STAI, SSS-V, BIS) were assessed using multivariate ANOVAs.

Correlations between behavioral performance [frequencies and reaction times of high risk (2_9) and low risk (5_6) selections], hormone concentration and self-report questionnaire measures (SSS, BIS, BDI, STAI) were computed testing two-sided for negative, respective positive correlations.

Since progesterone (FO: $p = 0.007$, LU: $p = 0.326$; OC: $p = 0.893$) and estradiol (FO: $p = 0.811$; LU: $p = 0.002$; OC:

$p = 0.469$) levels were not normally distributed in the three female groups, we transformed the values taking the square root, which is an adequate tool to apply to right skewed data (Bortz, 1999). The transformed hormone values then were normally distributed (progesterone: FO: $p = 0.072$, LU: $p = 0.343$; OC: $p = 0.917$; estradiol: FO: $p = 0.589$; LU: $p = 0.063$; OC: $p = 0.747$) and thus were entered in further analyses. In the male group, testosterone concentration was normally distributed (morning: $p = 0.879$, afternoon: $p = 0.737$).

Following the study by Stanton et al. (2011), we distributed our female and male group into high and low testosterone groups via median split of testosterone concentration.

Pearson correlations were calculated to investigate the influence of sex hormone levels on the behavioral performance. To adjust for significant inter-hormonal correlations additional partial correlations were calculated, controlling for estradiol/progesterone influence on the correlation between performance and hormone levels, respectively. Moreover, estradiol:progesterone ratio was calculated and entered in the correlation analyses.

RESULTS

Figure 2 displays performance of all group comparisons and **Table 1** shows means and standard deviations of hormone concentration and neuropsychological parameters.

FEMALES

Hormone concentration

Females in the three groups showed significantly different progesterone levels [$F_{(2, 68)} = 12.700$, $p < 0.001$, part-eta sq. = 0.272]. *Post-hoc* analysis showed that LU females had significantly higher progesterone levels than both other groups (LU vs. FO: $p = 0.001$; LU vs. OC: $p < 0.001$). No group difference emerged for estradiol [$F_{(2, 68)} = 1.145$, $p = 0.324$]. **Table 1** (top section) shows means and standard deviations of hormone concentration.

Decision-making

Applying a mixed-model ANOVA with risk selection as within-subject factor and group (FO vs. LU vs. OC) as between-subject factor, we observed a significant risk selection effect

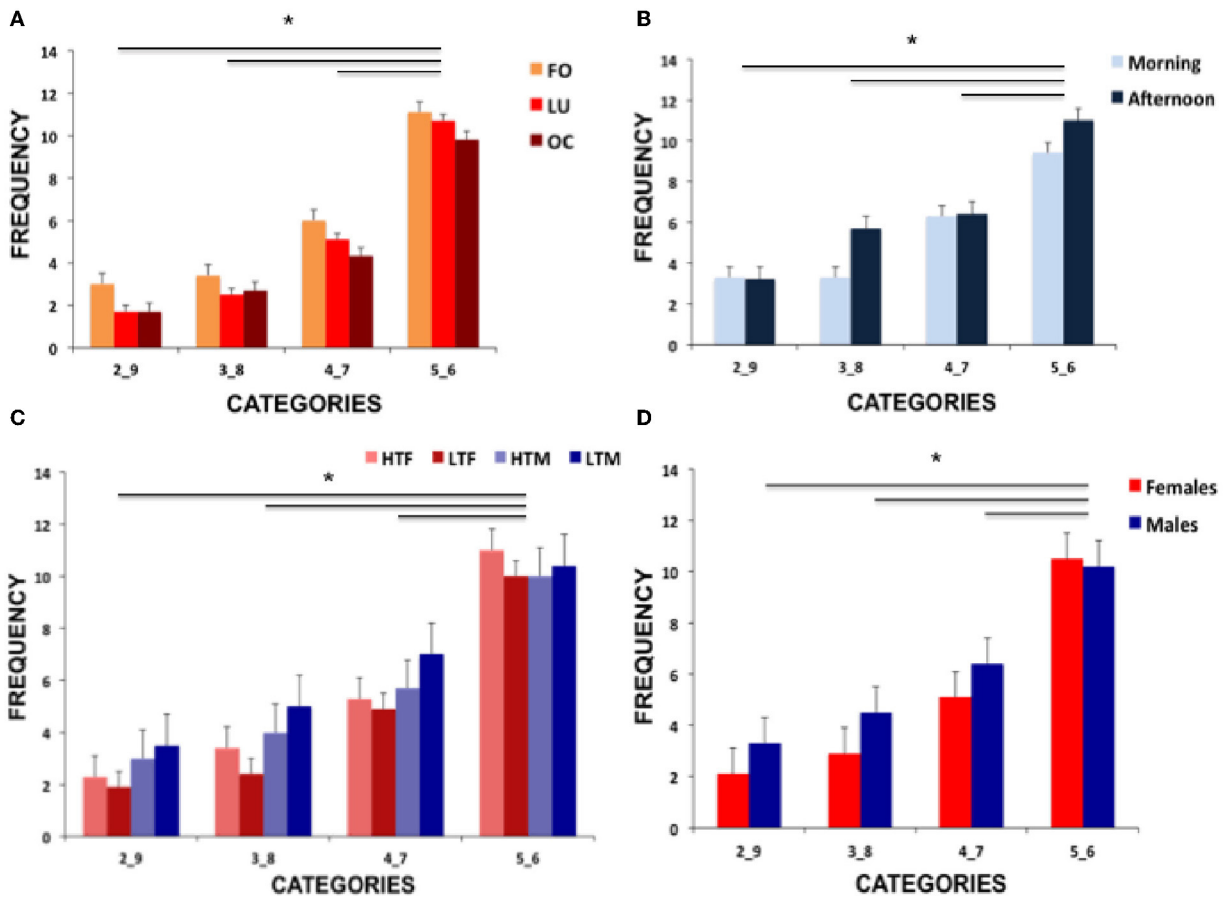

FIGURE 2 | Illustration of the results showing (A) frequencies of risk conditions for the three female groups (FO, follicular; LU, luteal; OC, oral contraceptive intake), (B) frequencies of the two male groups (morning and afternoon testosterone) and (C) frequencies of the high vs. low testosterone concentration females and males (HTF, high testosterone females; LTF, low testosterone females; HTM, high testosterone males; LTM, low testosterone males) and (D) comparison of performance of females and males. Significant differences are marked with an asterisk.

[$F_{(1.741,\ 118.393)} = 134.049$, $p < 0.001$, part-eta-sq. $= 0.663$], no significant group effect [$F_{(1,\ 68)} = 0.668$, $p = 0.516$] and no significant interaction [$F_{(1.741,\ 118.393)} = 0.321$, $p = 0.839$].

Post-hoc tests disentangling the significant risk selection effect revealed that the highest number of selections was present for the least risky parameters (5_6) and the lowest number of selections was present for the most risky parameters (2_9, all p-values < 0.019). See **Figure 2A** for illustration of results.

Regarding reaction times, we focused on the high (2_9) and low (5_6) risk conditions. The mixed-model ANOVA revealed no significant effect of risk selection [$F_{(1,\ 71)} = 0.214$, $p = 0.647$], no significant group effect [$F_{(1,\ 71)} = 0.874$, $p = 0.428$], and no significant risk-by-group interaction [$F_{(1,\ 71)} = 0.950$, $p = 0.398$].

Neuropsychological performance and questionnaire data

Females in the three groups did not differ in neuropsychological parameters (MWT-B: $p = 0.647$; TMT-A: $p = 0.769$; TMB-B: $p = 0.922$), or in the questionnaire data (all p-values >0.195). See also **Table 1** (top section) for detailed information.

Correlation analyses between behavioral performance and hormone concentration

Analysis of a significant association between behavioral performance (risk selection and reaction times) and hormone concentrations (estradiol, progesterone) revealed a significant correlation between progesterone and reaction time in the high risk condition in LU ($r = 0.471$, $p = 0.048$), indicating faster responses in females with lower progesterone concentration. No other significant correlations emerged. For details see **Table 2**.

Applying partial correlations did not reveal a significant correlation (all p-values > 0.054).

Relying on the estradiol:progesterone ratio revealed a significant correlation with low risk selection (HRG 5_6: $r = 0.657$, $p = 0.019$) as well as with reaction time in the high risk condition (HRG 2_9: $r = -0.530$, $p = 0.026$) again in LU. In FO and OC no correlation reached significance (all p-values > 0.107). For details please see **Table 2**.

Correlation analyses between behavioral performance and questionnaire data

In FO, trait anxiety correlated with high risk reaction time ($r = 0.577$, $p = 0.016$). In LU, the thrill and adventure score from the SSS-V ($r = 0.808$, $p = 0.004$) was significantly positively correlated with reaction time for the low risk selection. No other significant correlations emerged (all p-values > 0.051).

MALES

Hormone concentration

Testosterone levels were significantly different ($t = 2.355$, $p = 0.023$), with higher concentration in the morning group.

Table 2 | Overview on correlations between hormone concentrations and behavioral performance (selection as well as reaction times) for all groups.

	Selection		Reaction time	
	HGR 2_9	**HGR 5_6**	**HGR 2_9**	**HGR 5_6**
Follicular				
Estradiol	$r = -0.150$, $p = 0.505$	$r = 0.018$, $p = 0.936$	$r = 0.054$, $p = 0.855$	$r = 0.312$, $p = 0.299$
Progesterone	$r = -0.154$, $p = 0.493$	$r = -0.289$, $p = 0.192$	$r = -0.127$, $p = 0.666$	$r = -0.133$, $p = 0.665$
E:P	$r = 0.007$, $p = 0.976$	$r = 0.359$, $p = 0.101$	$r = 0.292$, $p = 0.312$	$r = 0.340$, $p = 0.256$
Luteal				
Estradiol	$r = -0.123$, $p = 0.550$	$r = 0.029$, $p = 0.888$	$r = -0.189$, $p = 0.626$	$r = -0.255$, $p = 0.507$
Progesterone	$r = -0.135$, $p = 0.510$	$r = -0.315$, $p = 0.117$	***r = 0.471, p = 0.048***	$r = 0.442$, $p = 0.234$
E:P	$r = 0.052$, $p = 0.802$	***r = 0.657, p = 0.019***	***r = −.530, p = 0.026***	$r = 0.400$, $p = 0.257$
Oral contraceptives				
Estradiol	$r = 0.103$, $p = 0.638$	$r = 0.316$, $p = 0.141$	$r = 0.113$, $p = 0.755$	$r = -0.282$, $p = 0.429$
Progesterone	$r = -0.125$, $p = 0.569$	$r = 0.072$, $p = 0.744$	$r = 0.111$, $p = 0.760$	$r = -0.337$, $p = 0.341$
E:P	$r = 0.223$, $p = 0.305$	$r = 0.267$, $p = 0.217$	$r = 0.037$, $p = 0.919$	$r = 0.109$, $p = 0.765$
Morning group M				
Testosterone	$r = 0.163$, $p = 0.468$	$r = -0.053$, $p = 0.814$	$r = 0.509$, $p = 0.075$	$r = 0.226$, $p = 0.457$
Afternoon group M				
Testosterone	$r = -0.281$, $p = 0.194$	$r = 0.025$, $p = 0.910$	$r = -0.113$, $p = 0.688$	$r = -0.263$, $p = 0.344$
High testosterone F				
Testosterone	$r = -0.028$, $p = 0.871$	$r = 0.072$, $p = 0.678$	$r = -0.097$, $p = 0.711$	$r = -0.298$, $p = 0.263$
Low testosterone F				
Testosterone	$r = 0.013$, $p = 0.942$	$r = 0.188$, $p = 0.280$	$r = 0.040$, $p = 0.882$	$r = 0.037$, $p = 0.891$
High testosterone M				
Testosterone	$r = 0.138$, $p = 0.541$	$r = -0.170$, $p = 0.450$	$r = 0.065$, $p = 0.834$	$r = -0.201$, $p = 0.511$
Low testosterone M				
Testosterone	$r = -0.097$, $p = 0.661$	$r = 0.051$, $p = 0.818$	$r = -0.283$, $p = 0.307$	$r = -0.332$, $p = 0.227$

Significant correlations are marked in bold.

Table 1 (middle section) shows means and standard deviations of hormone concentration.

Decision making

Applying a mixed-model ANOVA with risk selection as within-subject factor and daytime as between-subjects factor revealed a significant effect of risk selection [$F_{(1.849,\ 79.515)} = 29.568$, $p < 0.001$, part-eta-sq. $= 0.407$], no significant group effect [$F_{(1,\ 43)} = 0.623$, $p = 0.434$] and no significant interaction [$F_{(1.849,\ 79.515)} = 1.145$, $p = 0.320$]. *Post-hoc* analysis of the significant risk effect showed that high-risk selections were taken less often than the lower risk options (all p-values < 0.022).

Regarding reaction times of high and low risk selection, mixed-model ANOVA revealed no main effect of risk selection ($F_{(1,\ 26)} = 0.271$, $p = 0.607$], no main effect of group [$F_{(1,\ 26)} = 1.670$, $p = 0.208$], nor any interaction [$F_{(1,\ 26)} = 1.798$, $p = 0.192$]. See **Figure 2B** for results.

Neuropsychological performance and questionnaire data

Males did not differ in executive functioning (TMT-A: $p = 0.483$; TMT-B: $p = 0.710$) but in verbal intelligence (MWT-B: $p = 0.047$) with higher scores in the afternoon group. For details please see also **Table 1** (middle section). Re-running the repeated-measures ANOVA with MWT-B as covariate did not change the results in risk selection or reaction times.

Regarding questionnaire data, males in the morning group reported higher trait anxiety (STAI-T, $p = 0.037$) than the afternoon group. All other comparisons remained not significant (all p-values > 0.376). Including STAI-T scores as covariate in the repeated-measures ANOVA did not influence significance and direction of the reported effects.

Correlation analyses between behavioral performance and hormone concentration

Analysis of a significant association between behavioral performance (risk selection and reaction times) and testosterone concentration revealed no significant associations (all p-values >0.075). For details see **Table 2**.

Correlation analyses between behavioral performance and questionnaire data

While scores in sensation seeking, impulsivity, and depression were not correlated with behavioral performance, trait anxiety scores were correlated with high-risk selection ($r = 0.581$, $p = 0.002$) in the morning group. Correlations with state anxiety did not reach significance (all p-values > 0.052) and no significant correlation emerged for the afternoon group (all p-values > 0.068).

FEMALES vs. MALES

Hormone concentration

Applying the median split, 23 males were divided in the low testosterone group (HTM, 9 from the morning group, 14 afternoon group), 22 males in the high testosterone group (LTM, 13 from the morning group, 9 afternoon group), 35 females in the low testosterone group (LTF) and 36 in the high testosterone group (HTF). LTF vs. HTF and LTM vs. HTM differed significantly in their testosterone levels (both p-values < 0.001).

Table 1 (bottom section) shows means and standard deviations of testosterone concentration.

Decision making

Applying a mixed-model ANOVA with risk selection as within-subject factor and testosterone concentration as well as sex as between-subjects factor revealed a significant risk selection effect [$F_{(1.945,\ 217.857)} = 127.116$, $p < 0.001$, part-eta sq. $= 0.532$] but neither a testosterone effect [$F_{(1,\ 112)} = 0.001$, $p = 0.970$], nor a significant sex effect [$F_{(1,\ 112)} = 1.506$, $p = 0.222$] or interaction (all p-values > 0.142) occurred.

Regarding reaction times, no main effect of risk selection [$F_{(1,\ 56)} = 0.065$, $p = 0.800$] but a significant testosterone concentration effect [$F_{(1,\ 56)} = 4.039$, $p = 0.049$, part-eta sq. $= 0.067$] with faster responses in participants with low concentration and a trend for a sex difference [$F_{(1,\ 56)} = 3.899$, $p = 0.053$, part-eta sq. $= 0.065$] with faster reactions in females emerged. All interactions did not reach significance (all p-values > 0.298). See **Figure 2C** for results on testosterone groups and **Figure 2D** for results of females and males.

Neuropsychological performance and questionnaire data

Applying a multivariate ANOVA with testosterone concentration and sex as grouping factors revealed no significant main effect or interaction for verbal intelligence (MWT-B, all p-values > 0.256) or executive functioning (TMT-A, all p-values > 0.216; TMT-B, all p-values > 0.222).

Regarding questionnaire data, multivariate ANOVA again with sex and testosterone concentration as grouping factors demonstrated sex differences for the boredom susceptibility score [$F_{(1,\ 56)} = 8.945$, $p = 0.004$, part-eta sq. $= 0.085$], the thrill and adventure seeking score [$F_{(1,\ 56)} = 6.432$, $p = 0.013$, part-eta sq. $= 0.063$] as well as the total score of the sensation seeking scale [$F_{(1,\ 56)} = 9.389$, $p = 0.003$, part-eta sq. $= 0.091$] always with higher scores in males. Additionally for trait anxiety, females showed significantly higher scores than males (STAI-T, $F_{(1,\ 56)} = 8.421$, $p = 0.005$, part-eta sq. $= 0.079$]. For testosterone concentration no significant main effect ($p = 0.060$) or interaction with sex ($p = 0.103$) occurred and no other effect reached significance (all p-values > 0.060).

Correlation analyses between behavioral performance and hormone concentration

Analysis of a significant association between behavioral performance (risk selection and reaction times) and testosterone concentration in the separate groups (HTF, LTF, HTM, LTM) revealed no significant association (all p-values > 0.219). For details please see **Table 2**.

DISCUSSION

The present study aimed at analyzing the impact of menstrual cycle phase, diurnal testosterone variation, and testosterone concentration on decision-making relying on an evaluated task without learning effect, HRG (Haegler et al., 2010). Additionally, we investigated whether decision-making was associated with hormone concentration or personality and mood factors such as sensation seeking, impulsivity, depression or anxiety. This was realized by dividing the study cohort into three groups of females

(follicular, luteal, and pill-taking) testing for cycle effects. The effect of diurnal variation of male hormone concentration was studied in two male groups (morning and afternoon measurement). Following the approach by Stanton et al. (2011), we investigated the impact of testosterone concentration on performance parameters in females and males. Notably, all participants were students thus groups had similar age and educational background. Moreover, they did not differ in basic neuropsychological parameters including verbal intelligence and executive functions.

The following section will be divided into different parts discussing menstrual cycle effects, influence of diurnal variation of testosterone on decision-making in males and the impact of high vs. low testosterone concentration in females and males. Moreover, a more general discussion and limitations of the conducted study will be reported.

MENSTRUAL CYCLE AND DECISION-MAKING

Previous studies reported heterogeneous findings regarding the impact of menstrual cycle phase and hormone concentration on decision-making: studies relying on self-report data frequently reported a significant rise in risk-taking behavior when estradiol levels were high (Chavanne and Gallup, 1998; Bröder and Hohmann, 2003; Haselton and Gangestad, 2006; Pillsworth and Haselton, 2006; Sukolová and Sarmány-Schuller, 2011), Saunders and Hawton (2006) reviewed several studies on suicide attempts and suicidal behavior in women and observed that during phases of low estradiol levels non-fatal suicidal behavior is more frequent, while for example Reavis and Overman (2001) or van den Bos et al. (2007) did not report a significant impact of menstrual cycle phase on performance using the Iowa Gambling task. Here, we also observed no significant difference in risk selection or reaction time between follicular and luteal females. Moreover, in contrast to previous studies we included pill-taking females but no significant group effect emerged. Further analyses of impact of hormone concentration revealed two significant findings in LU: while progesterone concentration was negatively correlated with reaction time for high risk selection, estradiol:progesterone ratio was positively associated with low risk selection. Hence, while we see no general impact of menstrual cycle phase, correlations with hormone levels, and behavioral performance occurred only in LU, where particularly progesterone levels were higher.

Our findings indicate that during the luteal phase, females showed faster responses for high risk options when their progesterone levels were higher but chose low risk options more often the higher their estradiol:progesterone ratio.

Our findings thus point out two different aspects: (1) using computerized experimental paradigms to assess risk selection revealed no significant impact of menstrual cycle, while studies relying on self-report data do. Therefore, one has to question whether the constructs assessed with one and the other might be different, have distinct values and relevance for the participants and whether a bias between self-report and experimental behavior exists. (2) analysis of hormone concentration showed some associations with behavioral performance, particularly with progesterone, supporting previous findings of more risk taking behavior in the luteal phase with higher progesterone values.

Evidence has accumulated that progesterone and its metabolites (mainly allopregnanolone, 3α,5α-THP) are important neuroactive steroids, which influence social, cognitive, and physical performance (for review see Frye, 2009; Pluchino et al., 2013). During the luteal phase, circulating concentrations of pregnanolone and 3α,5α-THP are 2–4 times higher than during the follicular phase (Purdy et al., 1990; Wang et al., 1996; Genazzani et al., 1998; Sundström and Bäckström, 1998a,b), with highest concentrations in the hippocampus and midbrain regions (Bixo et al., 1997). Typically, decision-making tasks elicit activation of prefrontal regions but also hippocampus activation has been reported (Li et al., 2010, for review see van den Bos et al., 2013). However, up to now the impact of concentration of progesterone and its metabolites on behavioral performance and neural activation underlying decision-making is still unclear. Hence, pregnanolone and 3α,5α-THP might also influence cycle-mediated performance in these tasks thereby contributing to the findings observed in previous studies as well as ours.

Additionally, we observed a significant positive correlation between trait anxiety and reaction time in the high risk condition only in the follicular group, indicating longer reaction times with higher trait anxiety. Hence, females during the follicular phase took longer to decide for the high risk option. This fits nicely with that assumption that high trait anxiety is linked with risk-avoidant decision making, which has been shown before (Broman-Fulks et al., 2014; Pittig et al., 2014). However, for sensation seeking and impulsivity, we only observed sparse associations with decision-making behavior. Several studies linked risk taking behavior with these personality factors (e.g., Mishra and Lalumière, 2011; Popham et al., 2011), however, others also failed to observe these associations (Bayard et al., 2011). Again, one possible factor explaining this divergence is the methodological variety in how decision-making or risk-taking was assessed. To further investigate these associations, future experiments might want to combine several approaches in order to highlight divergences and communalities.

Regarding oral contraceptive intake we failed to report any significant effect or correlation of hormone concentration with behavioral performance. Several factors might have influenced our findings, such as the heterogeneity of oral contraceptives taken by our women or the lack of information on duration of intake. This should be further investigated in future studies.

DIURNAL VARIATION IN TESTOSTERONE AND ITS IMPACT ON DECISION-MAKING

Several studies linked testosterone concentration with risk-taking in that higher testosterone levels were associated with more risky behavior (e.g., Carney and Mason, 2010; Goudriaan et al., 2010). Despite the fact that we observed higher testosterone concentration in males measured in the morning compared to the afternoon, analysis of behavioral performance did not reveal a significant group effect and thus impact of the diurnal variation in testosterone on decision-making. Moffat and Hampson (1996) showed a significant difference in spatial processing between males tested at 8:15 vs. 10.15 a.m., with better performance in those with higher testosterone levels.

Interestingly, we observed a significant positive correlation of trait anxiety and high risk selection in the morning group, suggesting more risky decision making in males with higher trait anxiety. This finding contradicts a bulk of literature proclaiming less risk taking in high trait anxious individuals (Pittig et al., 2014). However, the three-way interaction of testosterone concentration, trait anxiety and decision-making performance in males has rarely been investigated, thus replications are necessary before conclusion can be drawn.

Additionally, in follicular females we observed a contradictory correlation, namely that higher levels of trait anxiety were associated with longer reaction times for risky selections, thus rather risk-aversive behavior. Sex differences in trait anxiety have been reported quite frequently, with higher values in females than males (Spielberger et al., 1983; McCleary and Zucker, 1991; Perkins et al., 2007). Moreover, sex-specific effects of trait anxiety on decision-making have also been reported before, suggesting sex-specific endophenotypes of anxiety which in turn affect cognitive functioning differentially (Visser de et al., 2010).

It remains an open question, which abilities are affected by diurnal variation and what role for instance seasonal variation of salivary testosterone concentration as shown by Stanton et al. (2011) plays regarding decision-making or more specifically, risk-taking.

TESTOSTERONE CONCENTRATION IN FEMALES AND MALES

Dividing females and males in groups with high and low testosterone concentration only revealed a significant testosterone effect for reaction times, with faster reactions in participants with lower testosterone concentration. As low risk options were selected most frequently by all participants this might partly support findings linking testosterone concentration and risk behavior. Interestingly, Stanton et al. (2011) showed that high-testosterone women and high-testosterone men made riskier choices than their low-testosterone counterparts of the same sex, and this effect was pronounced in women. Hence, the authors conclude that according to their findings high levels of testosterone are associated with willingness to incur greater risk in both sexes when using the IGT. In their review paper on sex differences in decision-making with a particular focus on studies using the IGT, van den Bos et al. (2013) resume that factors such as self-report vs. experimental modulation of risk taking behavior, acting in a group or acting alone or simply the fact that several studies investigating decision-making induced stress in females and males may lead to more risk-taking behavior in men. Notably, the authors conclude that previous data rather indicate no sex difference in immediate responses to emotional events, but only in the way these responses are regulated by for instance neuronal structures related to cognitive control.

GENERAL DISCUSSION

Sex differences in the propensity to take risks have been documented in a large number of questionnaire and experimental studies (e.g., van den Bos et al., 2012). In a meta-analysis by Byrnes, Miller, and Schafer (1999) who reviewed over 150 papers on sex differences in risk taking, authors concluded that males are more likely to take risks than females. Notably, Figner and Weber (2011) pointed out that these sex differences in risk taking are domain-specific and can be explained by risk perceptions, which in turn are influenced by familiarity (Weber et al., 2005). Interestingly, once these differences in risk perceptions are taken into account, most of the sex differences in risk taking diminish as pointed out by Figner and Weber (2011). Here, we did not see a significant sex difference in risk taking as measured with the Haegler-Risk-Game (HRG). Following a domain-specific approach, it is hard to place the HRG, as there was no financial risk, no ethical risk, no recreational risk, no risk regarding health, or safety and no social decision risk, just the gambling risk with no economic consequences. Thus, we assume that risk perception was very low in females and males probably contributing to the lack of a general sex difference.

A potential influencing factor of the existing decision-making tasks is that they cannot be executed repeatedly without excluding a learning effect. Therefore, in the current study we relied on a novel computerized decision-making task in which participants had to make decisions between contingencies (Haegler et al., 2010). Due to the lack of winning strategy, the HRG can be played repeatedly without a learning effect. As learning behavior is modulated by hormone concentration particularly in the luteal phase (Andreano and Cahill, 2009), this might partly explain why we did not obtain significant group differences, instead only correlations with hormone concentration in the luteal phase.

Additionally, the study context also influences risky decision-making, with less consistent findings in laboratory settings (as in the study) as in field experiments (Eckel and Grossman, 2008). Although risky decision-making might be less consistent due to a laboratory setting, it has been discussed quite openly, that contextual conditions may introduce additional heterogeneity due to a gender interaction effect (Krajnik et al., 2014). Even in an animal model the effect of the experimenters sex on the baseline response in an androstadienone experiment, which is supposed to act as a chemosignal in humans, has recently been observed (Sorge et al., 2014). Also, another study in humans investigating the same compound, reported that the setting, the manner, and by whom the experiment was conducted played a role in perception (Lundström and Olsson, 2005). Especially for sex hormones it cannot be completely exluded that the experimenter collecting the samples might have an impact.

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Effects of estrogen on higher-order cognitive functions in unstressed human females may depend on individual variation in dopamine baseline levels

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INTRODUCTION

The gonadal steroid hormone estrogen (i.e., estradiol) seems to modulate higher order cognitive processes driven by dopamine (DA) such as learning, reward processing, working memory (WM), and inhibitory control (Hampson, 1990a,b; Maki et al., 2002; Caldu and Dreher, 2007; Dreher et al., 2007; Gasbarri et al., 2008; Colzato et al., 2010, 2012; Jacobs and D'Esposito, 2011). However, it is important to note that sex steroid hormones have been shown to impact several other neurotransmitter systems, including gamma-aminobutyric acid (GABA). Indeed, in healthy women the cortical GABA levels declines from the follicular phase (FP) to the mid luteal and late luteal phases (Epperson et al., 2002). In a recent review, Sinclair et al. (2014) suggested that the adolescent brain is shaped by the interaction between estrogen and glucocorticoids with a specific impact on DA neurotransmission. The focus of the present opinion article is not on glucocorticoid-estrogen interactions but on estrogen effects on higher-order cognition in unstressed human females.

High levels of estradiol are accompanied by increases in the attentional blink (Hollander et al., 2005) and in interference in the Stroop color-word task (Hatta and Nagaya, 2009), indicating reduced cognitive control. Moreover, the reactivity of the reward system is augmented in women during the midfollicular phase when estrogen is unopposed by progesterone (Dreher et al., 2007).

Previous studies have shown that gender differences in DA-modulated higher-order cognitive processes are restricted to a particular phase of the female menstrual cycle: the late FP, in which the estrogen level is high. Growing evidence suggests that the dopaminergic system seems to be particularly strongly affected by estrogen. After estrogen enters the brain, it is converted into cathecol estrogen, which has been considered to inhibit the catechol O-methyltransferase (Ball et al., 1972), an enzyme responsible for the degradation of DA in prefrontal cortex (PFC). Several studies have pointed out that the estrous cycle is related to augmentation in DA release associated with high levels of estrogen in rodents (Di Paolo et al., 1986; Becker et al., 2001; Dazzi et al., 2007; for review see Becker, 1999) and in monkeys (Czoty et al., 2009). Moreover, as pointed out by Czoty et al. (2009), receptor autoradiography studies have revealed that D2 receptor densities can raise in the presence of natural elevations in estrogen during the estrous cycle and after exogenous estrogen administration (Pazos et al., 1985; Di Paolo et al., 1988; Bazzett and Becker, 1994; Becker, 1999; see Di Paolo, 1994).

Interestingly, the ventral tier of the midbrain sends its DA projections to the dorsal and lateral parts of the PFC, while the dorsal tier sends its DA projections primarily to the ventral striatum, which projects strongly to ventrolateral and ventromedial PFC (Cools, 2006). As suggested by Miller (2000), reward information may be mediated by the dopamine-mediated innervation of PFC from a group of cells situated in the midbrain ventral tegmental area (VTA). Inhibitory control of behavior and thoughts seems to be driven by the frontal/basal-ganglia system. Finally, DA levels in PFC are related to the maintenance of WM information (Cools, 2006). Given these links between estrogen, DA, and PFC, it should not be surprising that PFC-depending functions, like inhibitory control, WM, and reward processing, are particularly affected by the menstrual cycle.

INDIVIDUAL BASELINE LEVELS OF DA MAY EXHIBIT DIFFERENTIAL SENSITIVITY TO ESTROGEN

In some previous studies, estrogen seems to have modulated cognitive processes in opposite directions or in unreliable ways. For example, studies have found improved verbal working memory (Rosenberg and Park, 2002) and better performance on a test of implicit memory (Maki et al., 2002) when the estrogen level was high, while others found high levels of estrogen to have a negative effect on delayed matching-to-sample working memory task (Gasbarri et al., 2008). Jacobs and D'Esposito (2011) were the first to suggest that inconsistencies in the literature linking WM and estrogen (Maki et al., 2002; Rosenberg and Park, 2002; Gasbarri et al., 2008) may be explained by taking baseline levels of DA into account. Indeed, these authors showed that the direction of the effect estrogen has on WM depends on indices of baseline DA (as assessed by the genetic variability associated with the COMT Val^{158}Met genotype).

We suggest that not only for WM, but for all cognitive processes related to DA, the effect of estrogen might depend on individual variation in baseline DA function, which follows an "Inverted-U"-shaped function. Indeed, neurotransmitters such as DA often relate to performance in a nonlinear fashion, with the best performance related to a medium level, while higher levels are likely to be counterproductive (Muly et al., 1998; Goldman-Rakic et al., 2000). This effect is explained by the existence of GABAergic interneurons with D1 receptors and inhibitory input to cortical pyramidal cells, which are related to cognitive performance. At moderate levels of dopamine release the function of these pyramidal cells (but not of the interneurons) is enhanced, which leads to better performance as compared to lower levels. But at high levels of dopamine release, the GABAergic inhibitory interneurons also get excited and start projecting the neurotransmitter GABA onto the pyramidal cortical cells. This provides them with inhibitory input, leading to impaired performance (Goldman-Rakic et al., 2000). Consistent with this picture, the impact of most dopaminergic agonist drugs is modulated by individual differences: increasing the dopamine level is likely to be beneficial for individuals whose level falls short of the optimal level but to impair the performance of individuals with medium (optimal) or high levels (Cools, 2006).

We speculate that different individuals may have different baseline levels of DA and may therefore exhibit differential sensitivity to the positive and negative effects of estrogen. Given that estrogen is associated with higher DA turnover rates, if estrogen affects the DA functioning in driving a particular cognitive function, we would expect a cognitive beneficial effect in the late FP (i.e., with the highest level of estrogen) for individuals with a low DA baseline level. In contrast, we would expect a cognitive detrimental effect in the late FP for individuals with an already optimal baseline level. That is, low baseline levels of DA, which are in general accompanied by poor cognitive performance, may be improved by high levels of estrogen. In contrast, high baseline levels of DA, commonly related to good cognitive performance, may be impaired by estrogen.

Colzato and colleagues showed, in two independent samples, that late FP was associated with both *less* efficient inhibitory output control (Colzato et al., 2010) and *more* efficient inhibitory input control (Colzato et al., 2012). Of course, we cannot exclude that this dissociation simply reflected the independence of input and output control (Johnston et al., 1995). However, if our idea that the effect of estrogen on all DA-driven cognitive processes depends on individual variation in baseline DA is correct, it is a real possibility that individual differences have modulated our previous findings. If so, it is reasonable to assume that our first study (Colzato et al., 2010) tapped a sample with an already optimal DA baseline level while the second (Colzato et al., 2012) happened to assess a sample with low DA baseline levels.

MARKERS OF DA BASE LEVELS

The direct assessment of DA function in humans is only possible by means of positron emission tomography (PET) so far, which is, however, very expensive and highly invasive due to radioactive contamination and arterial blood sampling (Volkow et al., 2009). An ideal index of DA base levels, also used by Jacobs and D'Esposito (2011), is genetic variability related to levels of DA, which is nonetheless still a costly procedure.

Interestingly, DA can be found in high concentration in the amacrine and interplexiform cells of the retina (Bodis-Wollner and Tzelepi, 1998; Witkovsky, 2004). Abnormal color discrimination has been reported for several neuropsychiatric conditions underlying al dopaminergic functions, such as Parkinson's and Huntington's disease, Tourette syndrome, ADHD, and cocaine use (Paulus et al., 1993; Pieri et al., 2000; Melun et al., 2001; Tannock et al., 2006; Hulka et al., 2013). Roy et al. (2003) suggested that color vision impairment points to a central hypodopaminergic state. Very recently, color vision has been found to predict the efficiency in resolving response conflict given that both are driven by dopamine (Colzato et al., under revision). This raises the possibility that individual color discrimination performance predicts individual differences in sensitivity to the positive (i.e., enhancing) and negative (i.e., unfavorable) effects of estrogen. For example in tasks assessing cognitive control and adaptation one would expect benefits (e.g., better goal regulation in the face of response conflict) in the late FP for individuals with poor color discrimination but a detrimental effect (e.g., poorer goal regulation) in individuals with optimal color discrimination.

Another interesting measure of DA functioning is the spontaneous eyeblink rate (EBR), a well-established clinical indicator (Karson, 1983; Shukla, 1985; Blin et al., 1990; Taylor et al., 1999). Patients with DA-related dysfunction show atypical patterns: EBRs are elevated in in schizophrenia patients (Freed, 1980) but reduced in recreational cocaine users (Colzato et al., 2008b) and Parkinson's patients (Deuschel and Goddemeier, 1998). Moreover, pharmacological studies in nonhuman primates and humans have shown that DA agonists, such as apomorphine, and antagonists increase and decrease EBRs, respectively, (Blin et al., 1990; Kleven and Koek, 1996). Similarly to color vision, EBR has also been found to predict DA-driven cognitive processes (e.g., Dreisbach et al., 2005; Colzato et al., 2007, 2008a, 2009). Accordingly, EBR, in interaction with the individual genetic setup, should predict individual differences in sensitivity to the positive and negative effects of estrogen. In particular, we would expect cognitive benefits in the late FP for individuals with low EBR but impairments in individuals with an average/high EBR.

It might be particularly informative to use proton magnetic resonance spectroscopy (1H-MRS) and plasma levels of homovanillic acid (HVA) to trace the impact of estrogen on the DA system. 1H-MRS permits to measure the concentration of particular chemicals, based on subtle differences in the resonance of the protons they contain. This technique has been successfully applied in the past to reflect changes in dopamine pathways (Moore et al., 2006) and to investigate the effect of dopaminergic treatment on the cortex (Lucetti et al., 2007). In contrast to PET, 1H-MRS does not use invasive radioactive tracers and it is way less expensive. 1H-MRS allows measuring brain metabolites including creatine (Cr), inositol (Ino), and glutamate and

glutamine (Glx), and the ratio between them. Because protons experience different shielding effects from the surrounding electrons in different molecules, their resonance varies from one type of molecule to another. In the late FP we would expect increased brain DA and, accordingly, a modulation of the Glx-to-Cr and Glx-to-Ino ratio.

Homovanillic acid (HVA) is a metabolite of DA which is typically decreased in repetitive behavior disorders (Lewis et al., 1996) and it may be altered in in disorders of catecholamine metabolism. For example, monamine oxidase-A deficiency can cause decreased HVA values, while a deficiency of dopamine beta-hydrolase (the enzyme that converts dopamine to norepinephrine) can cause elevated HVA concentrations. Accordingly, HVA values should predict individual differences in sensitivity to the positive and negative effects of estrogen. In particular, we would expect cognitive benefits in the late FP for individuals with low HVA values but impairments in individuals with an average/high HVA values.

SUMMARY

We propose that future studies investigating the effect of estrogen on DA-driven higher order cognitive processes should take into account individual differences in DA base levels. The existing research on the role of estrogen in higher order cognitive processes has been mainly "effect"-driven, and thus only shown *that* estrogen can have an effect without explaining *how* it modulates cognitive processes and why some people benefit more than others. To get a better understanding of the underlying mechanism and the interplay between estrogen, dopaminergic supply, and cognitive functioning it is mandatory to develop a comprehensive, detailed model of how estrogen modulates higher order cognitive processes in healthy humans.

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Sex hormones affect neurotransmitters and shape the adult female brain during hormonal transition periods

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Sex hormones have been implicated in neurite outgrowth, synaptogenesis, dendritic branching, myelination and other important mechanisms of neural plasticity. Here we review the evidence from animal experiments and human studies reporting interactions between sex hormones and the dominant neurotransmitters, such as serotonin, dopamine, GABA and glutamate. We provide an overview of accumulating data during physiological and pathological conditions and discuss currently conceptualized theories on how sex hormones potentially trigger neuroplasticity changes through these four neurochemical systems. Many brain regions have been demonstrated to express high densities for estrogen- and progesterone receptors, such as the amygdala, the hypothalamus, and the hippocampus. As the hippocampus is of particular relevance in the context of mediating structural plasticity in the adult brain, we put particular emphasis on what evidence could be gathered thus far that links differences in behavior, neurochemical patterns and hippocampal structure to a changing hormonal environment. Finally, we discuss how physiologically occurring hormonal transition periods in humans can be used to model how changes in sex hormones influence functional connectivity, neurotransmission and brain structure *in vivo*.

Keywords: estrogens, progesterone, neurotransmitters, plasticity, hormonal transition periods

INTRODUCTION

Over the last decades, several lines of research have extended the pivotal actions of ovarian hormones such as estrogen and progesterone outside of the reproductive tract. The brain represents an important target for estrogen and progesterone effects. Both hormones provide specific neuroendocrine conditions through which brain structure and function are modulated across a woman's life span. The trophic effects of ovarian hormones emerge early in brain development and remain throughout adolescence (Juraska et al., 2013) and adulthood (Wise et al., 2008). Many of these actions occur in brain regions involved in learning (Hu et al., 2007) and memory (Liu et al., 2008), emotion (Amin et al., 2006), motivation (Sakaki and Mather, 2012), motor control (Horstink et al., 2003), and cognition (Berman et al., 1997). Furthermore, several lines of evidence support a main impact of sex hormones on brain development and plasticity (Marino et al., 2006). Specific structural effects of estrogen and progesterone include neurite outgrowth and synaptogenesis (Haraguchi et al., 2012), dendritic branching (Cooke and Woolley, 2005) and myelination (Garcia-Segura and Melcangi, 2006).

GENERAL MECHANISMS

Both estrogen and progesterone act via classical genomic receptors as well as non-classical membrane-associated receptors (see **Table S1** for overview on main genomic and non-genomic signaling properties). The classical estrogen receptors (ERα/β) (Gundlah et al., 2001; Mitra et al., 2003) and progesterone receptors ($PR_{A/B}$) (Brinton et al., 2008) are highly expressed in brain areas involved in emotion and cognition, such as amygdala and hippocampus. Ovarian hormones can act on multiple receptor types, such as voltage-gated ion channels, including $GABA_A$(Gulinello et al., 2001), NMDA (Foy et al., 1999), serotonin (Sumner and Fink, 1998) and dopamine (Becker, 1990) receptors. While these genomic actions of sex hormones require a comparably long time—from minutes to hours—and are limited by the rate of protein biosynthesis, non-genomic modulation of the membrane receptors is mostly faster and requires only milliseconds to seconds (McEwen, 1991; Cornil et al., 2006). Both estrogen and progesterone exert acute effects on synaptic physiology through the activation of multiple intracellular signaling pathways (Minami et al., 1990; Krebs et al., 2000; Wu et al., 2005), including the MAPK/ERK and the Akt pathway which are both part to a non-genomic signaling cascade linked to the promotion of cell survival (Singh, 2001). A distinct progesterone-binding protein different from the classical PR was identified as a membrane protein, known as 7TMPR, which mediates non-genomic actions via second-messenger cascades (Zhu et al., 2003a,b). However, genomic and non-genomic actions of hormones may also be coupled, so the distinctions are not as clear-cut as was first thought (Vasudevan and Pfaff, 2008).

It is noteworthy that most cellular effects of ovarian hormones have important roles in cell survival, apoptosis, function, and brain development and may act as critical neuroregulatory, neurotropic, and neuroprotective factors in brain physiology and pathological conditions of the brain. Effects on structure and function of the brain have been documented.

EFFECTS ON STRUCTURE

For instance, fairly recent studies have shown that progesterone, progestin or progestin metabolites could have the capacity to induce or inhibit neuroplastic changes by preventing microglia from releasing harmful free radicals (Muller and Kerschbaum, 2006) or by stimulating myelin production (Baulieu and Schumacher, 2000). Furthermore, ovarian hormones also exhibit profound effect on neurotrophins such as brain-derived neurotrophic factor (BDNF). BDNF has been shown to play a key role in neuronal survival, in promoting neuronal regeneration following injury and regulating neurotransmitter system (Scharfman and MacLusky, 2006; Sohrabji and Lewis, 2006). Estrogen treatment seems to increase BDNF expression in several brain regions including hippocampus, amygdala and cortex (Zhou et al., 2005), and has been shown to decrease the risk for neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Sohrabji and Lewis, 2006).

However, much translational work remains to be done, and it is not clear which of these mechanisms are most relevant to clinical use. As an example, evidence for the beneficial effects of progesterone on cognitive outcome following traumatic brain injury has recently been reviewed as promising (Ma et al., 2012)—emphasizing the need to further promote this research direction.

Although estrogen and progesterone target multiple regions in the brain (Brinton et al., 2008), one brain region that has been the focus of many studies investigating potential neurotropic effects of these hormones is the hippocampus, a brain region associated with various memory functions (Bliss and Collingridge, 1993; Adams et al., 2004). Both, acute estrogen and progesterone treatment have been shown to increase synapse density and spine formation in hippocampal structures in rodents, respectively (Woolley and McEwen, 1993). However, the generative effects of progesterone seem to disappear after chronic treatment. Furthermore, progesterone has also been shown to down-regulate estrogen-induced synapses when added to estrogen-administration chronically (Woolley and McEwen, 1993). Thus, the duration and combination of ovarian hormone supplementation seems to be essential for its neuroplastic effects on brain structures, such as the hippocampus. Therefore, the overall modulatory effect of ovarian hormones is more complex than simple mechanistic processes of up- and down-regulation of expression patterns in isolated brain regions.

In humans, evidence for hormone-dependent modulatory effects on brain structure stems from hormonal replacement therapy (HRT) studies. The importance of ovarian hormones has lead to its use as HRT, primarily to treat menopausal/postmenopausal symptoms such as hot flashes and night sweat. However, structural and functional changes associated with HRT regimes have sparked a heightened interest in ovarian hormone effects in the human body. For instance, brain structure changes due to HRT seem to be most prominent in the hippocampus. Women using HRT showed an increased hippocampal volume compared to men and women who never used HRT (Lord et al., 2008).

Not only are exogenous sex steroid hormones likely to influence the structure and function of the hippocampus, but variable sex steroid levels across the female lifespan have also been associated with alterations in hippocampal structure (Adams et al., 2004; Galea et al., 2008).

EFFECTS ON FUNCTION

Beyond structural changes mediated by HRT, ovarian hormone supplementation is also known to have prominent effects on mood and cognitive functioning in domains such as working memory and executive control. In general, positive (Hogervorst et al., 2000; LeBlanc et al., 2001; Rice and Morse, 2003; Weber et al., 2014) and negative (Rapp et al., 2003; Shumaker et al., 2003) effects on cognition have been reported for HRT. In virtue of these contradictory findings, HRT is currently much under debate. However, it seems that timing (MacLennan et al., 2006) and dose (Rice, 2002) are critical aspects of how the impact of HRT unfolds.

A woman's lifespan is characterized by major hormonal transition periods beginning with rising estrogen level during puberty (Angold et al., 1998), high estrogen levels during pregnancy and rapid falls postpartum (Galea et al., 2001), declining levels during perimenopause (Cohen et al., 2006a) and low levels postmenopausal. Intriguingly, these major shifts in sex hormone levels seem to be paralleled by the incidence rates of mood disorders such as unipolar depression (**Figure 2**). According to the monoamine hypotheses of depression (Hindmarch, 2002), depressed mood seems to be accompanied by alternations in neurotransmitter functioning and transmission. Ovarian hormones are known to exhibit modulatory effects on synaptic transmission. These modulatory effects can be achieved by altering the responsiveness of postsynaptic receptors (Yankova et al., 2001; Maejima et al., 2013) or the presynaptic release of neurotransmitters (Yokomaku et al., 2003). The alternation of both mechanisms largely affect the neurochemical systems involved in healthy emotional and cognitive control, such as dopaminergic, serotonergic, glutamatergic and γ-aminobutyric acid (GABA)-ergic systems.

Along with the major hormonal transition periods, subtle changes in endogenous sex hormones, as occur during the monthly cycle, have also been associated with changes in mood (Backstrom et al., 2014). A subgroup of women, however, suffers from clinical level of premenstrual mood changes called premenstrual dysphoric disorder (PMDD), a condition that has recently been included in the DSM-V (Epperson et al., 2012a). PMDD core symptoms include anxiety, irritability and depressed mood (Epperson et al., 2012a). As the absolute levels of ovarian hormones do not seem to differ significantly in PMDD women and healthy controls (Backstrom et al., 2003), one hypothesis proposes that it is a heightened vulnerability of the central nervous system to normal ovarian function and physiological changes (Schmidt et al., 1998; Huo et al., 2007) rather than hormone imbalance, that predisposes women to PMDD. In the pathology

of PMDD, the normal functioning of predominantly two main neurotransmitter system, the serotonergic and dopaminergic system seems to be impaired.

The present review aims to summarize recent findings on the interaction between ovarian hormones and various neurotransmitter systems in the brain. The second part of the review focuses on the discussion of these findings in the context of neuropsychiatric diseases that display a substantial sexual dimorphism, such as affective disorders and Alzheimer's disease.

SEX HORMONES AND THEIR INTERACTION WITH NEUROTRANSMITTER SYSTEMS—POTENTIAL MECHANISMS FOR SEX HORMONES TO EFFECT BRAIN STRUCTURE AND FUNCTION

The classification of the main neurotransmitter systems are summarized in **Table S1**.

SEX HORMONES AND GLUTAMATE INTERACTION

Glutamate acts as the main excitatory neurotransmitter in the CNS and is a proximal regulator of cognitive domains such as learning and memory (Gazzaley et al., 1996; Foy et al., 1999; Riedel et al., 2003). The integration of glutamatergic transmission is fundamental for normal cognitive functioning and mental health (Schwartz et al., 2012; Abdallah et al., 2014). The cortical glutamate projections are organized in descending and ascending pathways that project throughout much of the telencephalon (**Figure 1A**). The impact of ovarian hormones on the glutamatergic system has been studied extensively, especially in cell cultures (Yokomaku et al., 2003) and animal models (Bethea and Reddy, 2012; Wei et al., 2014). Both stimulatory (Yokomaku et al., 2003) and inhibitory (Smith et al., 1987a) effects of ovarian hormones have been reported. In rodents, several mechanisms through which ovarian hormones may influence glutamatergic neurotransmission have been proposed: progesterone has been shown to suppress the excitatory glutamate response in a dose-dependent fashion (Smith et al., 1987a), while estrogen exhibits facilitating effects on glutamate transmission (Yokomaku et al., 2003). A physiological dose of progesterone in ovariectomized rats has been reported to reduce glutamate-response by 87% via attenuation of non-NMDA receptors (AMPA, Kainate) (Smith et al., 1987b). The magnitude of the attenuation seems directly proportional to the progesterone dose. Whereas progesterone mainly impacts non-NMDA receptors (Smith et al., 1987a), the mechanisms underlying estrogen effects on cognition are related to NMDA glutamate receptors. Estrogen has been shown to promote an increase in NMDA receptor subunit expression (Gazzaley et al., 1996; Adams et al., 2004), binding sites (Woolley et al., 1997) and neuronal sensitivity to synaptic input mediated by NMDA glutamate receptors (Rudick and Woolley, 2001; Smith and Woolley, 2004). The blockade of NMDA receptors with antagonists attenuates the effects of estrogen on neuronal correlates of memory, such as long-term potentiation (LTP) (Brinton et al., 1997; Foy et al., 1999). Moreover, estrogen facilitates the spine-maturation process (Hao et al., 2006). A plethora of animal studies have shown that estrogen with and without progesterone increases dendritic spines through the up-regulation of AMPA (Liu et al., 2008; Kramar et al., 2009) and NMDA receptors (Woolley et al., 1997) in the hippocampus and prefrontal cortex (PFC) (Hao et al., 2006). In addition, ovariectomy reduced synaptic markers in these regions (Gould et al., 1990; Hao et al., 2006).

Beside ovarian influence on spine density, precursors for progesterone (such as pregnenolone) seem to have complex effects on glutamate release itself that depends on the developmental period, brain region and functional state (Zheng, 2009): in the hippocampus (Meyer et al., 2002) and in the prefrontal cortex (Dong et al., 2005), which are brain regions of high relevance to memory and executive control, progesterone precursors have been shown to impact spontaneous glutamate release that may contribute to the maturation and/or maintenance of synapses. The latter might point toward the well-established neuroprotective effects (Mattson et al., 1997; Wang et al., 2001) of ovarian hormones from insults such as oxidative stress and glutamate excitotoxicity.

In addition to these cellular effects, recent studies report that the interaction between estrogen and glutamate can affect cognitive domains such as working memory and executive function under harmful conditions. Brain regions hypothesized to underlie these cognitive domains—such as the PFC and hippocampus—seem largely dependent on normal estrogen signaling to counter insults such as stress: In a repeated stress paradigm, Wei et al. (2014) found a beneficial effect of endogenous estrogen on glutamate receptors in the PFC in female rats compared to male rats. The authors propose that detrimental effects of repeated stress are present in females when estrogen signaling is blocked, whereas detrimental effects are blocked in males when estrogen signaling is activated. In particular, blocking estrogen synthesis enzyme aromatase with formestane in PFC revealed stress-induced glutamatergic deficits and memory impairment in female rats (Wei et al., 2014). Thus, these results suggest that the female rodent PFC has an endogenous capacity to generate estrogen that provides protection against subchronic repeated stress. How gender differences in response to stressors are modulated by hormonal status is extensively reviewed by Cohen and Yehuda (2011). In addition to endogenous estrogen levels, also exogenous administered estrogen seems to increase the resilience to stress and preserve hippocampal functioning in rats (Bredemann and McMahon, 2014).

Although animal studies assessing electrophysiological, biochemical and behavioral markers for sex hormonal impacts on the glutamatergic system provide useful insights on underlying mechanisms, extrapolation to humans is difficult. Some of the beneficial effects of estrogen on cognitive function have also been shown in humans: premenopausal women who were treated with a gonadotropin releasing hormone analog which chemically suppressed ovarian function experienced significant deterioration of mood and worsening of performance in working memory tasks (Grigorova et al., 2006). In this study, the estrogen levels dropped to postmenopausal values implicating that low endogenous levels of estrogen might impair normal cognitive functioning.

Although this study report positive effects of estrogen on working memory and executive function, the results are inconclusive (Grigorova and Sherwin, 2006), and the advisability of hormonal replacement is still much under debate. However, a

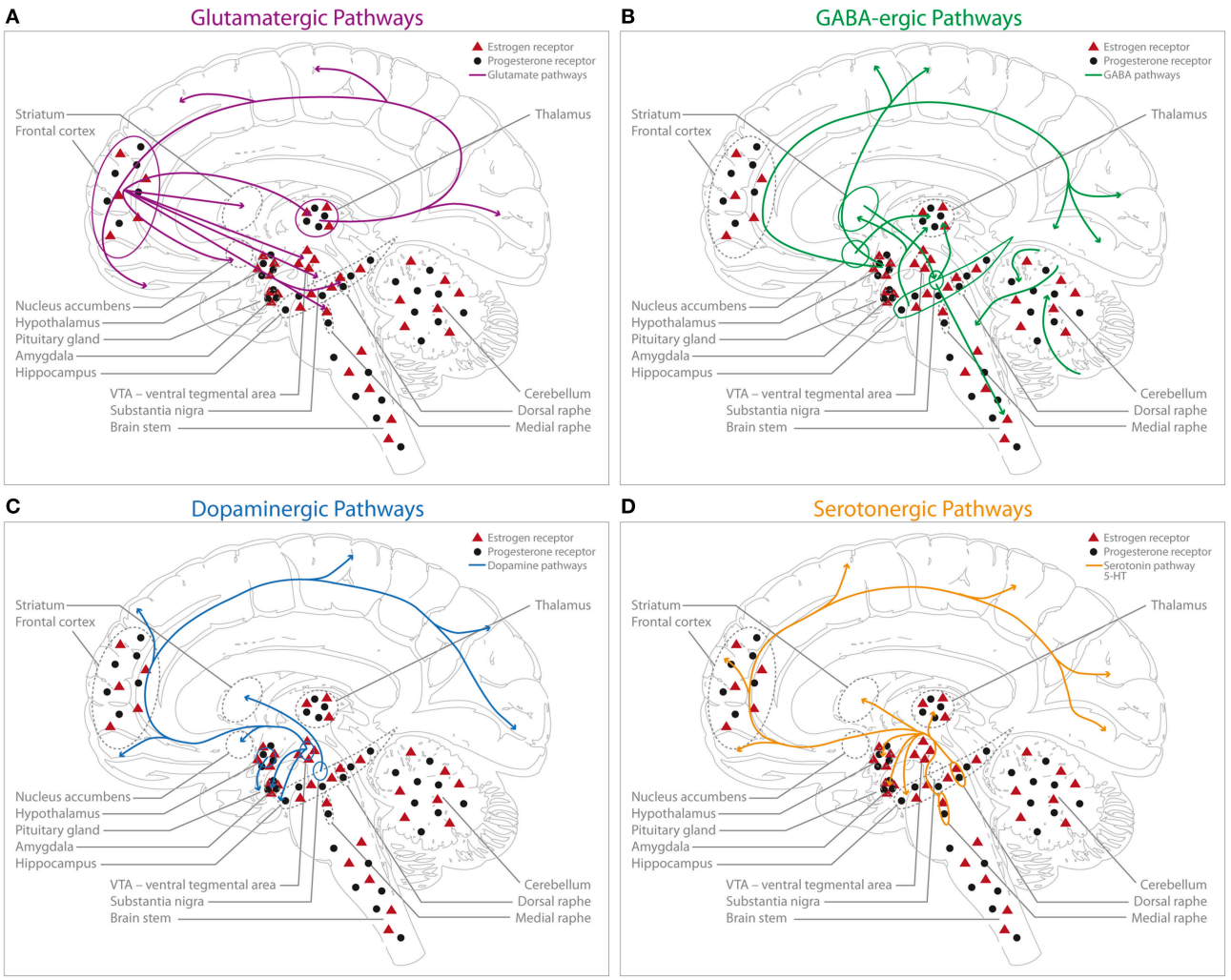

FIGURE 1 | Schematic representation of the main human central glutamatergic system (A, purple), GABAergic system (B, green), dopaminergic system (C, blue) and serotonergic system (D, orange) including a schematic display of estrogen (ERα and ERβ combined) and progesterone receptor distribution in the human brain. No distinction is made between ERα and ERβ sub-specification for this schematic display, however the localization for those subtypes can differ [e.g., so far no evidence could be gathered supporting ERα expression in the dorsal raphe nucleus (DRN) (Sugiyama et al., 2010)], however there have been reports on ERβ expression in primate DRN in the midbrain (Gundlah et al., 2001; Sugiyama et al., 2010). Estrogen (red triangles) receptors are predominantly present in cerebellum, VTA, hippocampus, amygdala, and frontal cortex; as well as in the raphe nuclei of the midbrain (Gundlah et al., 2001; Osterlund and Hurd, 2001; Mitra et al., 2003; Perlman et al., 2005; Sugiyama et al., 2010). Progesterone (filled circles) receptor expression could be shown in the amygdala, midbrain, brain stem, hippocampus, cerebellum and frontal cortex with no apparent restrictions to specific cell types (Bethea, 1993; Gundlah et al., 2001). **(A)** The cortical glutamatergic projections can be separated in five main pathways (Schwartz et al., 2012): (1) from prefrontal to brainstem areas (dorsal/medial raphe, VTA, substantia nigra); (2) from prefrontal cortex to striatum and nucleus accumbens; (3) the thalamocortical pathway, from thalamus to cortical pyramidal neurons; (4) inverse projections from cortex to thalamus; and (5) intra-cortical glutamate projections. **(B)** GABAergic projections are widely distributed throughout the brain. Main projections can be found originating in the striatum to the substantia nigra and the brain stem. Further projections innervate the thalamus from the substantia nigra (Fino and Venance, 2010). Moreover, GABAergic projections originate from (1) hypothalamus to occipital cortex and parietal cortex; (2) from hippocampus to thalamus and striatum, and (3) from nucleus accumbens to thalamus. The cerebellum is also highly innervated by GABAergic projections. **(C)** The cortical dopaminergic pathways build four distinct pathways (Felten and Shetty, 2010): (1) the mesolimbic pathway; with projections from VTA to limbic structures, such as the nucleus accumbens, hippocampus, amygdala and prefrontal cortex, (2) the mesocortical pathway; with projections from the VTA to cerebral cortex, (3) the nigrostriatal pathway; with connections between substantia nigra and striatum, (4) the tuberoinfundibular pathway; with projections starting from the hypothalamus to the pituitary gland. **(D)** The majority of serotonergic projections originates from the dorsal and median raphe nuclei, innervating the amygdala, hypothalamus, thalamus, striatum, cerebral cortex and hippocampus (Felten and Shetty, 2010): (1) The medial raphe predominantly projects to the frontal cortex and the hippocampus (Hornung, 2003) and (2) the areas of the dorsal raphe mainly innervate the thalamus, striatum and cerebral cortex (Geyer et al., 1976).

large number of studies with behavioral testing during hormonal transitions, such as the menstrual cycle or postmenopause, point toward an estrogen-dependent improvement in memory (Epperson et al., 2012b; Hampson and Morley, 2013), but the neurochemical pathways underlying these changes remain to be identified. A useful approach to assess glutamate release *in vivo* in humans might be a pharmacological stimulation of the glutamatergic system with positron emission tomography (PET) using a glutamate-receptor radioligand. In conjuction with MR-Imaging it could link glutamate release to brain activation during, i.e., working memory tasks. Such methods should lead to a better understanding of the interaction between sex hormones and glutamatergic neurotransmission.

SEX STEROID HORMONE AND GAMMA-AMINOBUTYRIC-ACID INTERACTION

Gamma-aminobutyric acid (GABA) is the most abundant and widely distributed inhibitory neurotransmitter in the CNS (Sieghart and Sperk, 2002; Marshall, 2008). GABAergic neurotransmission through interneurons is known to modulate local neuronal circuits via, for example, activation of dopaminergic (Dewey et al., 1992) and serotonergic neurons (Andrade et al., 1986). GABAergic interneurons can be differentiated into two types, each acting via its receptor-subtype (**Table S2**). GABA receptors are highly distributed in cortical, hippocampal, thalamic, basal ganglia and cerebellar structures (**Figure 1B**).

$GABA_A$ receptors mediate major inhibitory GABAergic actions in the CNS and are putative sites for ovarian hormone effects (Backstrom et al., 2011, 2014). Whereas estrogen seems to suppress GABA inhibitory input (Murphy et al., 1998a), progesterone and its neuroactive metabolites (allopregnanolone, pregnanolone) seem to facilitate GABAergic transmission through their action at $GABA_A$ receptors (van Wingen et al., 2008; Deligiannidis et al., 2013). Particularly, allopregnanolone acts like a positive modulator and potentiates the inhibitory action of GABA by increasing channel openings of the GABA-gated chloride channels (Rupprecht, 1997) and augmenting other inhibitory neuronal responses to GABA (Smith, 1991). This facilitation of GABA-mediated Cl^- current can result in inhibitory effects on neuronal function.

The interaction of progesterone and its neurosteroids with neuronal $GABA_A$ receptors is significantly influenced by the subunit composition of the receptor, local metabolism and phosphorylation (Belelli et al., 2006; Backstrom et al., 2014). Especially the subunit composition seems to play a crucial role in inhibitory neurotransmission and its effects on a larger scale such as mood and cognition (Backstrom et al., 2014). Animal studies indicate a relationship between changes in α4 and δ subunits of $GABA_A$ receptors and anxiogenic effects of allopregnanolone (Gulinello et al., 2001). Alternations in both GABA receptor subunit expression and anxiety behavior reflect a complex temporal pattern following sustained exposure to progesterone metabolites: An increase in hippocampal expression of the α4 subunit is seen to correlate with increased anxiety after 48 h exposure to allopregnanolone (Hsu et al., 2003). Paradoxically, in high concentrations, progesterone and its neurosteroids are also known to be anxiolytic, sedative, and antiepileptic, both in animals and humans (Backstrom et al., 2014). Allopregnanolone naturally fluctuates across the female menstrual cycle, with its highest concentration in the luteal phase when progesterone is high and estrogen is low (Backstrom et al., 2014). In women with PMDD, progesterone withdrawal associated with allopregnanolone increase in the luteal phase has been linked to changes in mood (Epperson et al., 2012a). As absolute levels of ovarian hormones do not seem to differ in PMDD women compared to healthy controls (Backstrom et al., 2003), it is proposed that a heightened vulnerability of the central nervous system to normal ovarian function predisposes women to PMDD.

Beyond its influence on mood, progesterone and its metabolites also seem to impact the memory and learning domains. Animal studies could show that allopregnanolone can inhibit neural activity in the CA1 and the dental gyrus area of the hippocampus (Landgren et al., 1998). The magnitude of the allopregnanolone inhibition is dependent on the phase of the rodent estrus cycle, with its maximum in the luteal phase. In humans, acute progesterone or allopregnanolone administration has been shown to impair face recognition and episodic memory in healthy women, while endogenous allopregnanolone level does not seem to impact their memory and learning. However, women suffering from PMDD showed impaired working memory in N2 and N3 back tasks during their symptomatic phase (Yen et al., 2012). Thus, healthy women seems to show memory impairments when progesterone and its metabolites are administered exogenously, while women with PMDD are more vulnerable to endogenous fluctuations of progesterone and allopregnanolone across the menstrual cycle.

Based on all these findings, Backstrom and colleagues recently hypothesized that an increase of the α4, β, δ subunit composition, together with a heightened vulnerability toward elevated allopregnanolone levels, could be key factors in the progesterone withdrawal model of PMDD (Backstrom et al., 2014). As PMDD heightens the risk for other kinds of depression, such as postpartum depression, a detailed understanding of the underlying mechanisms of PMDD might foster therapeutic approaches and thereby subsequently promote female mental health.

SEX STEROID HORMONE AND DOPAMINE INTERACTION

Dopamine (DA) is a key neurotransmitter that is implicated in motor control (Sealfon and Olanow, 2000; Dluzen and Horstink, 2003), learning (Daniel et al., 2006), motivation (Becker, 2009), reward (Hikosaka et al., 2014), decision-making and working memory (Jacobs and D'Esposito, 2011; Uban et al., 2012). Brain areas that show rich dopaminergic innervation include the striatum, substantia nigra and hypothalamus (Sealfon and Olanow, 2000); typically four main pathways are described for the dopaminergic system (**Figure 1C**).

Sex hormones can impact dopaminergic neurotransmission via a multitude of mechanisms (synthesis, release, turnover and degradation, pre-and postsynaptic receptors, transporters, for details see **Table S2**). Stimulating (Becker, 1990, 2000; Thompson and Moss, 1994; Becker and Hu, 2008), as well as inhibiting (Disshon et al., 1998; Watson et al., 2006; Morel et al., 2009) effects of estrogen on dopaminergic neurotransmission have been documented. These conflicting findings are not surprising when

considering the many aspects that can influence the impact of estrogen on the DA-system such as dose and time of testing, mode of administration, duration of exposure, and time after exposure (Di Paolo, 1994). Most experts agree that estrogen has an overall facilitating effect on dopaminergic neurotransmission (Sanchez et al., 2010; Jacobs and D'Esposito, 2011; Uban et al., 2012; Rey et al., 2014). For progesterone-modulating effects, it seems that, in addition to the previously mentioned aspects, priming with estrogen can also influence the impact of progesterone on dopaminergic transmission: *in vitro* (Dluzen and Ramirez, 1984; Cabrera et al., 1993) and *in vivo* (Becker, 1990) experiments support a stimulating effect on DA-release when rats where pre-exposed to estrogen whereas no such effects could be observed in non-estrogen treated rats.

Still much work remains to be done to improve our understanding of how these findings translate to the human brain, and ultimately link to human behavior and potentially pathology. Nevertheless, there are several examples for recent evidence converging from animal and human work that emphasize the relevance of this line of research to female mental health. Recent studies report on the interaction between estrogen and dopamine on cognitive domains, such as decision-making (Uban et al., 2012), fear extinction (Rey et al., 2014) and memory bias (Quinlan et al., 2013). The authors conclude that estradiol biases decision-making toward smaller, more accessible rewards (Uban et al., 2012), that a low-estrogen state during fear extinction is detrimental for an optimal freezing suppression after extinction, which is mediated by D1-receptor signaling (Rey et al., 2014) and that memory bias is mediated by the interaction of estradiol and dopamine in the dorsal striatum (Quinlan et al., 2013). In particular, the latter two studies argue that the effect of estrogen depends on individual variation in baseline DA function and endorse the concept of estrogen-dopamine interactions to mirror an inverted U-shaped curve, a model that has also been tested by several other studies (Williams and Goldman-Rakic, 1995; Gjedde et al., 2010). Thus, optimal signaling may depend on the levels of estrogen to best interact with dopamine levels in the median range for optimal striatal function and optimal performance during such a task.

This hypothesis has been tested in humans. Estrogen-DA interaction in PFC function during a working memory task has been linked to variations in the gene for catechol-o-methyltransferase (COMT), the enzyme that metabolizes synaptic dopamine (Jacobs and D'Esposito, 2011). The authors found *val/val* women to perform poorly with low estrogen levels (early follicular phase) and improve with rising estrogen levels (late follicular phase), whereas *met/met* women show the opposite pattern. Best performers were women with high COMT (low DA) just prior to ovulation (high estrogen levels), and women with low COMT activity (high DA) during menses, further supporting the inverted U-shaped action of DA. Based on these findings, the authors propose that the effect of estrogen on cognitive performance could be either beneficial or detrimental depending on COMT genotype and COMT enzymatic activity (Jacobs and D'Esposito, 2011). While these concepts require further testing, they offer interesting perspectives for the planning of HRT in postmenopausal women.

Several neuropsychiatric pathologies that display a substantial sexual dimorphism have been linked to abnormal dopaminergic function, such as schizophrenia (Brunelin et al., 2013), Parkinson's (Dluzen and Horstink, 2003; Horstink et al., 2003; Sanchez et al., 2010) or Alzheimer's disease (Reeves et al., 2009). Thus, a better understanding of the interaction between sex hormones and dopaminergic neurotransmission could help to improve pharmacological treatment regimens for these diseases and significantly impact women's mental health.

SEX HORMONES AND SEROTONIN INTERACTION

The serotonergic system serves a multitude of roles, most prominently balancing mood (Martinowich and Lu, 2008). Several serotonergically mediated physiological functions are tightly linked to steroid hormones such as sexual behavior or stress response (Biegon, 1990). The highest expression of serotonergic neurons can be found in the dorsal and medial raphe nuclei of the midbrain with ascending fibers projecting to frontal cortex, striatum, thalamus, amygdala, hypothalamus and hippocampus (**Figure 1D**). Serotonin (5-HT) receptor subtypes belong to G-protein coupled or ligand-gated ion channels and 15 different subtypes have been characterized (**Table S2**), which is why disentangling specific neurochemical action for each subtype has been described as complex (Murphy et al., 1998b).

It is also difficult to provide a detailed characterization of serotonergic action in the CNS and how it can be influenced by sex hormones. A multitude of factors plays into serotonergic neurotransmission such as: endogenous levels of serotonin, more than 15 neuroreceptor-subtypes, individual receptor expression, binding and affinity, the functional polymorphism of the serotonin transporter (5-HTT), intracellular protein levels, synthetic enzymes, and the prominent sexual dimorphism (Rubinow et al., 1998). The 5-HTT transports 5-HT back from the synaptic cleft into the presynaptic neuron, where the neurotransmitter is predominantly metabolized by Monoamine Oxidase A (MAO-A). Furthermore, the serotonergic system is a main target of steroid hormones, cytokines, neuropeptides and trophic factors, all of which impact the generation and efficacy of serotonergic neurotransmission (McEwen, 2002; Bethea and Reddy, 2012).

Estrogen has been reported to have potent serotonin-modulating properties from the level of neurotransmitter synthesis via the regulation of tryptophan hydroxylase (Lu et al., 1999) and degradation of 5-HT to the density and binding of 5-HT receptors (Bethea et al., 2002). The effect of estrogen on serotonin expression seems to depend on several factors such as: receptor subtype, brain area and in case of estrogen treatment also on the duration of treatment. On the one hand, estrogen administration has been found to increase tryptophan hydroxylase mRNA (TPH, serotonin synthesizing enzyme) (Pecins-Thompson et al., 1996; Berman et al., 2006); 5-HT_{2A} mRNA levels in brain areas relevant for the control of mood, mental state and cognition (Sumner and Fink, 1998) and 5-HTT mRNA when administered for a longer period (Smith et al., 2004). On the other hand, estrogen treatment has also been observed to decrease mRNA related to serotonergic neurotransmission. For instance, 5-HT1B autoreceptor mRNA in dorsal raphe (Hiroi and Neumaier, 2009) and MAO-A mRNA and activity (Gundlah et al., 2002) are decreased after estrogen

treatment. Furthermore, acute estrogen administration decreases 5-HTT mRNA levels (Pecins-Thompson et al., 1998) and 5-HT_{1A} mRNA levels and binding. The latter effect disappears after a more chronic treatment-regimen (Osterlund et al., 1999, 2000). Thus, assigning the effects of estrogen on serotonin to a homogenous functional class of stimulation or inhibition seems not to be feasible.

Progesterone has been suggested to increase serotonergic neurotransmission via the regulation of the expression of serotonin-related genes and proteins (Bethea et al., 2002; Smith et al., 2004; Sanchez et al., 2005). Chronic progesterone treatment seems to decrease 5-HT_{1A} receptor expression in rats (Biegon et al., 1983), a finding that has also been reported for progesterone in combination with estrogen (Henderson and Bethea, 2008). In this study, the authors showed a decrease of $5HT_{2C}$ receptor expression when progesterone was added to the estrogen administration in macaques (Henderson and Bethea, 2008).

Both, estrogen and progesterone have been demonstrated to modify the serotonergic responsivity to selective serotonin reuptake inhibitors (SSRI)-administration (Benmansour et al., 2012). Experiments in rhesus macaques suggest that the sex steroids interact with the functional polymorphism of the 5-HTT to influence SSRI treatment response (Michopoulos et al., 2011). In humans, a recent study found an association between functional polymorphic region of the serotonin transporter gene (5-HTTLPR) and antidepressant efficacy in non-menopausal women (Gressier et al., 2014). Non-menopausal women with at least one copy of the long allele showed better antidepressant efficacy than those who were homozygous for the short allele (Gressier et al., 2014). Intriguingly, no differences were found in menopausal women. Menopause is typically associated with estrogen withdrawal suggesting that the hormonal status is critical for antidepressant efficacy. These findings add to previous studies suggesting that menopausal women gain less benefit from antidepressant treatments compared to women during their reproductive years (Pinto-Meza et al., 2006; Pae et al., 2009). In conclusion, the interaction between ovarian hormone levels, age and genotype appear to modulate serotonergic reactivity in females.

INFLUENCE OF SEX STEROID HORMONES ON NEURAL CIRCUITS: FROM HEALTH TO VULNERABILITY AND DISEASE

THE IMPACT OF SEX HORMONE FLUCTUATION ON THE HEALTHY BRAIN

Several lines of evidence from neuroimaging indicate modulatory effects of sex steroid hormones on different structural and functional brain connectivity parameters such as white matter structure (De Bondt et al., 2013), gray matter structure (Protopopescu et al., 2008), and overall network connectivity (Hausmann et al., 2002; Weis et al., 2008; Weis and Hausmann, 2010; Thimm et al., 2014).

On the overall brain level, it has been proposed that ovarian hormones facilitate both cortico-cortical and subcortico-cortical functional connectivity, whereas testosterone seems to decrease subcortico-cortical functional connectivity, but increases functional connectivity between subcortical brain areas, as reviewed by Peper et al. (2011). In line with this hypothesis, high levels of endogenous estradiol and progesterone have been observed to raise functional communication between both hemispheres (Hausmann et al., 2002), a mechanism that has been speculated to underlie sex differences in functional cerebral asymmetries (FCA) (Hausmann et al., 2002; Weis and Hausmann, 2010).

Ovarian hormone levels fluctuate on a monthly basis in women. Thus, studying functional and structural brain organization across the menstrual cycle represents a feasible approach to address the question whether sex hormones can influence functional and structural connectivity. A voxel-based morphometry (VBM) MRI study found gray matter density to increase in the right anterior hippocampus and decrease in the right dorsal basal ganglia in the late-follicular phase compared to late luteal phase (Protopopescu et al., 2008). Pletzer et al. report significant differences in gray matter density between naturally cycling women and women using oral contraceptives (OC), observing increased gray matter volume in prefrontal and temporal regions in OC users (Pletzer et al., 2010). Furthermore, the potential impact of hormonal contraception on brain structure does not seem to be limited to gray matter, and white matter tracts seem to be altered by OC use as well, especially in the fornix (De Bondt et al., 2013).

Sexual dimorphism in functional networks of the brain, such as the default mode network (DMN), a network that is proposed to underlie physiological processes unrelated to any particular thought (Gusnard et al., 2001), has been well established (Peper et al., 2011; Tian et al., 2011). However, few neuroimaging studies have investigated the extent to which sex hormones can influence the behavior of functional networks at rest. A recent study exploring functional connectivity of the anterior DMN and the executive control network (ECN) found differences in intrinsic connectivity between OC users and naturally cycling women, and it reported connectivity to differ most between groups in the left angular gyrus, the middle frontal gyrus, and the anterior cingulate cortex (ACC) (Petersen et al., 2013). Within the group of naturally cycling women, the follicular phase was associated with an increase in connectivity with the ECN relative to the luteal phase in the right ACC (Petersen et al., 2013).

A better understanding of functional and structural connectivity changes in the context of sex steroid fluctuations seems crucial to establish neurobiological models of neuropsychiatric diseases that display a strong sexual dimorphism, such as depression (Kessler et al., 1993; Kessler, 2003). Linking functional connectivity measures to neurochemical mechanisms during hormonal transition periods can be viewed as one of the next frontiers in the field of neuroimaging.

HOW SEX HORMONE FLUCTUATION CAN REPRESENT A PERIOD OF HEIGHTENED RISK FOR THE BRAIN: FROM VULNERABILITY TO DISEASE

Women have a lifetime prevalence rate for depression 1.5–3 times higher than men (Kessler, 2003). This distinction between the sexes is most prominent during the reproductive years (Soares and Zitek, 2008). For women, hormonal transitions across their lifespan represent periods of elevated vulnerability to development of mood disorders: elevated and fluctuating sex hormones seem to predispose women to mood-disturbance, beginning with

a heightened risk of developing a depressive episode following puberty (Soares and Zitek, 2008).

Pregnancy has been speculated to offer some protection against depression (Ko et al., 2012), including findings of lower suicide rates during pregnancy (Hawton, 2000; Oates, 2003). However, other work suggests that there is no difference in the prevalence rates of depression between pregnant and non-pregnant women (Vesga-Lopez et al., 2008). In a recent extensive study that screened 10,000 postpartum women, one third of screen-positive postpartum depressed women reported the onset of their depressive symptoms during pregnancy (Wisner et al., 2013). Pregnancy has also been discussed to confer vulnerability upon women who are already at risk of developing a depressive illness (Cohen et al., 2004, 2006b). Furthermore, recent findings (Rallis et al., 2014) indicate that symptom levels of depression, anxiety, and stress vary over the course of pregnancy, with women experiencing fewer symptoms during the middle of the pregnancy. Increased depressions scores early in pregnancy seemed to be predictive of later depression symptoms, especially postbirth (Rallis et al., 2014).

In summary, the current evidence does not unequivocally support pregnancy itself to pose an increased risk of developing depression, nor does it clearly identify this period as protection for the majority of women from mood disorders. Factors that might contribute to this ambiguity include that clinical assessment of depression during pregnancy and the postpartum period is complicated, because many of the typical depressive symptoms (disruption of sleep and appetite) are unavoidable during pregnancy and postpartum (Marcus, 2009), and that there may be a distinct pattern of reactivity in women who have never had a depressive episode before, vs. women who are already at risk (Cohen et al., 2004, 2006b).

To better address these challenges in the future, several strategies could be implemented: (1) A routine and serial screening process for depressive symptoms, administered by trained midwives, throughout pregnancy, and during the immediate and the extended postpartum period that focuses on the psychological symptoms, such as changes in mood, the tendency to ruminate, and anxiety. Studies suggest that screening with simple checklists and short screening tools, such as the Edinburgh Depression Scale, can already result in highly efficient screening for postpartum depression (PPD) (MacArthur et al., 2002). (2) Standardized psychoeducation on depression during pregnancy, postpartum blues, and PPD needs to become an integral part of standardized prenatal and postpartum care to help destigmatize PPD and facilitate the process of seeking proper diagnosis and adequate treatment. (3) Based on converging data from 17,000 women, individually tailored psychosocial and psychological intervention is a very promising way to prevent PPD, with provision of intensive, professionally-based postpartum home visits, telephone-based peer support, and interpersonal psychotherapy among the most effective strategies (Dennis and Dowswell, 2013). Thus, within the psychoeducational process, emphasis should be placed on individual risk evaluation, as well as on the wide range of intervention options available to women who fall within the spectrum of pregnancy-related or postpartum mood disorders, covering aspects from psychosocial support, psychotherapy, and psychopharmacology.

In this section, we focus on three examples of hormonal transition across the adult female lifespan: (a) the postpartum period, (b) the perimenopausal period, and (c) the more subtle fluctuation of sex hormones during the menstrual cycle.

Postpartum period

With the loss of the placenta, estrogen levels decrease 100–1000-fold during a period of a few days (Nott et al., 1976; O'Hara and Swain, 1996; O'Hara et al., 2000). This dramatic hormonal change is likely to induce a cascade of signaling that also affects the brain. A PET study investigating the neurochemistry of the female brain in the immediate postpartum period found a substantial whole-brain increase in MAO-A in the brain in the first week postpartum compared to women who had not recently been pregnant (Sacher et al., 2010). MAO-A is an enzyme that metabolizes monoamines, such as serotonin, dopamine and noradrenaline. A significant increase in MAO-A has been proposed to be predictive for the recurrence of major depressive disorder (Meyer et al., 2006). These findings in humans are in line with the inverse relationship between estrogen levels and MAO-A which has been observed in cell lines (Ma et al., 1995), as well as in rat (Luine and McEwen, 1977; Chevillard et al., 1981) and macaque (Gundlah et al., 2002; Smith et al., 2004) models. The acute estrogen drop within the first week postpartum has been proposed to trigger the subsequent MAO-A peak that could explain the depressed mood that a majority of mothers experience during this time (Sacher et al., 2010). Elevated MAO-A levels have also been found in prefrontal cortical regions and areas of the ACC in women with PPD and in women who do not meet criteria for a full PPD but report postpartum crying (Sacher et al., 2014). Thus, the interaction between estrogen and MAO-A seems to be a crucial factor in balancing postpartum mood. Given the current lack of prevention strategies for PPD, translation of biological concepts to facilitate the normalization of MAO-A levels in the brain, including potentially attenuating the acute hormonal withdrawal that can precede such an MAO-elevation, represents a promising line of research.

Further evidence that monoaminergic imbalance contributes to the development and/or severity of PPD symptomatology stems from neurochemical investigations of the serotonergic and the dopaminergic system: similar to depressed patients, PPD-patients with a history of non-postpartum depressed episodes seem to display a decrease in 5-HT1A receptors in the anterior cingulate and mesiotemporal cortices (Moses-Kolko et al., 2012). Postpartum status and unipolar depression have also been associated with lower striatal D2/3 receptor binding in postpartum and unipolar depressed women compared to healthy women who were not postpartum (Moses-Kolko et al., 2012). In summary, the postpartum period seems to be characterized by several monoaminergic alteration processes that are highly relevant to the regulation of mood and emotional processing.

GABA has also been implicated in the neurobiology of PPD: In a human pilot study, reduced occipital cortex GABA levels have been reported in parallel with decreased allopregnanolone levels during the postpartum period, irrespective of PPD diagnosis (Epperson et al., 2006). In animal models of postpartum

depression, abnormalities of GABA receptors (R delta and gamma 2 subunits) have been observed and discussed as being related to the substantial progesterone decline postpartum (Maguire and Mody, 2008). Further work in animal models mimicking the hormonal environment of the postpartum period revealed a characteristic behavioral phenotype with vulnerability to helplessness, increased anxiety, and aggression that has been associated with differences in expression of several key genes, such as 5-HTT, BDNF, GABA-A receptor type 4 (Suda et al., 2008). Data obtained from elegant animal models for PPD based on estrogen withdrawal across parturition (Galea et al., 2001) and exposure to high cortisol levels (Brummelte et al., 2006) support changes in steroid hormonal environment to spark dramatic effects in spatial memory and hippocampal morphology. This work further strengthens the strong argument that can be made for the heightened plasticity of the postpartum brain that seems to be driven by a closely intertwined action between sex hormones and neurotransmitters.

Perimenopausal transition

The perimenopausal transition period marks the end of the reproductive years and is commonly defined as a decline of ovarian function based on reproductive endocrine and menstrual cycle changes (Harlow et al., 2003). Three key markers for the onset of perimenopause are: (1) 7-day or more change in the menstrual cycle length; (2) a change in menstrual flow amount or duration, or (3) amenorrhea lasting at least 3 months (Harlow et al., 2003). Moreover, especially the increase in FSH levels beside elevated LH levels seems to be linked to a decline in ovarian function (Harlow et al., 2003). With the loss of ovarian function and the associated fundamental changes in the hormonal environment, it is not surprising that this phase in a woman's life is accompanied by changes in eating (Hirschberg, 2012), metabolism (Wing et al., 1991; Lovejoy et al., 2008), sleep (Guidozzi, 2013), behavior (Copeland et al., 2006), mood (Cohen et al., 2006a), sexuality (Dennerstein et al., 2003), immune response (Gameiro et al., 2010), and cognitive function (Greendale et al., 2012).

The concept that the perimenopausal phase represents a vulnerability period for developing a depressive illness is supported by evidence for a high rate of new-onset major depressive episodes (MDE) during this time (Cohen et al., 2006a; Freeman et al., 2006). In a longitudinal, prospective cohort study, Cohen and colleagues found that women with no lifetime history of depression who enter the menopausal transition earlier have a significant risk of first onset of depression (Cohen et al., 2006a). Strikingly, women with a previous history of depression, who also reported the use of antidepressants, had nearly 3 times higher risk of an earlier perimenopausal transition compared to non-depressed women (Harlow et al., 2003). Thus, there seems to be an inverse relationship between depression and the inception of perimenopause. Early perimenopause increases the risk of severe mood disturbances, while a lifetime history of depression predisposes women to an early onset of perimenopause. Furthermore, studies by Young et al. and Harlow et al. indicate that depressed women have lower estradiol and higher LH and FSH level than non-depressed controls (Young et al., 2000; Harlow et al., 2003). Whether the decline in estrogen levels or the cyclic fluctuation of estradiol level, which may increase in the menopausal transition (Cramer et al., 2002), contribute to the occurrence of depressed mood remain controversial (Joffe and Cohen, 1998; Halbreich, 2000).

Similar to the postpartum estrogen drop, perimenopausal estrogen decline seems to relate to an up-regulation of MAO-A levels in the brain. Rekkas et al. have recently shown greater MAO-A binding in the prefrontal cortex during the perimenopausal transition phase compared with age-matched women during their reproductive years and during menopause (Rekkas et al., 2014). The authors did not find any association between MAO-A binding and physical criteria of perimenopause, such as menstrual cycle length, vasomotor symptoms or plasma follicle-stimulating hormone levels. They did, however, find a significant correlation between MAO-A binding and the tendency to cry (Rekkas et al., 2014), a psychological symptom that has been found as a subclinical phenomenon to occur during major shifts of sex hormonal environment (Sacher et al., 2010; Dowlati et al., 2014).

Depressive mood during the perimenopausal years may also be of particular relevance for the development of dementia given that depressive episodes have been shown to increase the risk of Alzheimer's disease in later life (Devanand et al., 1996) and that severity of depression has been observed to predict progression from mild cognitive impairment to Alzheimer's disease (Van der Mussele et al., 2014) and cognitive function in patients with central nervous system disease (van Reekum et al., 2000). Estrogen fluctuations during the perimenopausal phase have been discussed to influence mood and cognition via several mechanisms. In addition to promoting anti-oxidative states that can support cell survival, for instance via balancing MAO-A levels (Ou et al., 2006; Fitzgerald et al., 2007), estrogen has been reported to trigger increased 5-HT_{2A} receptor binding that has been speculated to reduce the amount of β-amyloid deposition, a marker for Alzheimer pathology (Nitsch et al., 1994). Thus, it could be hypothesized that the perimenopausal drop in estrogen decreases the beneficial effect of increased serotonin binding on β-amyloid deposition. In ovariectomized rats, acute administration of estradiol seems to have an antidepressant effect via slowing extracellular serotonin clearance involving ERβ and G-protein coupled receptor. Beyond this, estrogen can also block the effect of SSRIs at the 5-HTT via estrogen receptor alpha (Benmansour et al., 2012).

Until today, it has been an area of much debate whether women with depressive symptoms should be treated with HRT, antidepressants or both. Core menopausal symptoms are related to deficits in declarative memory (Woods et al., 2000), fine motor coordination (Bayer and Hausmann, 2010), and feelings of depression (Hay et al., 1994; Freeman et al., 2006) and anxiety (Kessler, 2003; Faravelli et al., 2013; Soares, 2014). Several reviews and meta-analysis suggest small positive effects of HRT on verbal memory, attention, and reasoning (Hogervorst et al., 2000; LeBlanc et al., 2001; Rice and Morse, 2003; Weber et al., 2014). To generate a positive effect on cognition, the age of HRT onset seems to be crucial. Women receiving HRT earlier seem to improve their cognitive performance compared to either older HRT-treated women or untreated women (MacLennan

et al., 2006). This report is supported by neuroimaging findings suggesting increases in hippocampal size following estrogen treatment in postmenopausal women (Eberling et al., 2003). Animal studies further emphasize the age-related effects of estrogen treatment: Adams and colleagues report an up-regulation of both NMDA receptors and dendritic spines on CA1 pyramidal neurons in the hippocampus of young adult female rats, while aging rats respond to estrogen with the up-regulation of the NMDA receptor R1 subunit expression, solely (Adams et al., 2001). This neurochemical mechanism might be a starting point to understand the increased vulnerability of the aging hippocampus and decreased efficiency of HRT when administered later in menopausal transition or menopause and subsequently attenuated cognitive performance. Furthermore, perimenopausal estrogen depletion and greater activity of MAO-A are risk factors for Alzheimer's disease (Burke et al., 2004). When estrogen therapy is used early in menopausal transition, it can protect against dementia (Zandi et al., 2002).

To which extent mood is affected by HRT remains controversial. Estrogen has multifaceted neuromodulating effects and a particular emphasis has been placed on the interaction with the serotonergic system for some of the potential antidepressant benefits estrogen use in HRT may have for women during specific windows of time: Some studies suggest that estrogen might be useful to target perimenopausal (Schmidt et al., 2000; Soares et al., 2001), but not postmenopausal (Morrison et al., 2004).

Evidence from neuroimaging findings to link estrogen and the serotonergic system in humans are still relatively sparse. Animal data support ovariectomy to decrease 5-HT_1 binding (Biegon and McEwen, 1982), 5-HT_{2A} binding and expression (Sumner and Fink, 1993), and 5-HT transporter binding sites and expression (McQueen et al., 1997; Sanchez et al., 2013). These findings have been shown to be reversible with estrogen replacement therapy. However, interpretation of these animal data is constrained by the fact that they demonstrate specificity according to species, a certain brain area and a specific neurodevelopment stage. In humans, a recent PET study found no significant 5-HT_{1A} binding changes in postmenopausal women after estrogen or combined estrogen/progesterone treatment in any of the investigated brain regions including amygdala, ACC, hippocampus and prefrontal cortex (Kranz et al., 2014). A short course of estrogen can increase cortical 5-HT2A receptor density in healthy postmenopausal women's prefrontal regions (Kugaya et al., 2003). However, it still remains unclear to what extent these receptor density changes relate to clinical outcome as this study was done in healthy women and although the increase of 5-HT2A density was paralleled by improved performance in verbal fluency and in the trail making task, no significant changes in mood was observed (Kugaya et al., 2003). In contrast to short time estrogen administration, long-term estrogen treatment seems to be associated with lower 5-HT_{2A} receptor availability in hippocampus (Compton et al., 2008), a finding speculated to reflect increased activity within the serotonergic system leading to a down-regulation in postsynaptic 5-HT_{2A} receptor density. Again, the behavioral interpretation of these findings is difficult as a negative correlation between 5-HT_{2A} receptor availability and memory performance in postmenopausal females using long-term estrogen treatment (ET) has been found while the assessed depression scores did not differ between postmenopausal ET never-users and ET users (Compton et al., 2008). So far, no human *in vivo* PET study has investigated the effects of HRT on 5-HT-transporter binding. More translational work needs to be carried out before a conclusion regarding the role of estrogen replacement therapy as a potential mood enhancing strategy can be reached.

In summary, promising therapeutic approaches to improve perimenopausal mood and to counter depressive symptoms include strategies like inhibiting MAO-A, increasing multiple monoamines with antidepressants, and administering dietary amino acids that are precursors for the monoamines metabolized by MAO-A (Rekkas et al., 2014; Sacher et al., 2014). Regarding cognition, the use of HRT remains controversial. Many studies report only small effects on cognition such as verbal memory and attention (Hogervorst et al., 2000; Weber et al., 2014). These small effects on cognition and the potential side effects of HRT have to be carefully weighted. Unopposed estrogen cannot be used for extended periods due to the increased risk of endometrial hyperplasia (Furness et al., 2009) and malignancy (Weiderpass et al., 1999). To counter these potential side effects, progestin is normally added in oral contraceptive pills and HRT for endometrial protection (Weiderpass et al., 1999). However, the added progestin may worsen mood in some women (Backstrom et al., 2011). A concept also warranting further research is the targeting of estrogen receptors in a tissue-specific way using selective estrogen receptor modulators (Gambacciani, 2013; Mirkin et al., 2014). In conclusion, therapeutic use of HRT should be carefully considered in the context of perimenopausal symptom severity, age and prior history of HRT, dose, treatment combination, and timing of administration.

Menstrual cycle associated mood disturbances

The spectrum of severity in mood fluctuations throughout the menstrual cycle is wide and ranges from reports of less well-being in the premenstrual phase to severe clinical data on suicidal behavior: an evaluation of 44 studies in fertile women found a positive correlation between suicide attempts and menstrual phases that are characterized by low estrogen levels (Saunders and Hawton, 2006). In healthy women, some studies report negative premenstrual changes in mood as common and suggest that the majority of women of reproductive age describe a cycle-dependent increase in negative emotions, such as irritability, impulsivity, fear, and low mood (Halbreich et al., 2007), while other authors claim that there is no substantial evidence for any specific premenstrual negative mood syndrome in the general population (Romans et al., 2012). A subgroup of women, however, suffer from clinical levels of premenstrual mood changes called premenstrual dysphoric disorder (PMDD), a condition that has recently been included in the DSM-V (Epperson et al., 2012a). PMDD core symptoms include anxiety, irritability and depressed mood (Epperson et al., 2012a). Symptoms occur on average 2–3 days before onset of menses and resolve after the onset of menstruation (Backstrom et al., 2003). PMDD symptoms are limited to ovulatory menstrual cycles when the corpus luteum is present (Yen et al., 2013), thus it is reasonable to assume that female gonadal hormones play a causative role. However, no

consistent differences in hormonal fluctuations during the menstrual cycle between women experiencing clinical level PMDD and normal controls have been found (Backstrom et al., 2003).

Although the levels of sex hormones do not differ between PMDD and healthy women, there might be an altered genetic susceptibility to affective dysregulation induced by normal sex hormone levels. Preliminary genetic findings state an association between allelic variants in the estrogen receptor alpha gene (ESR2) and PMDD (Woods et al., 2000). As PMDD is a heritable disorder with non-Mendelian pattern (Bayer and Hausmann, 2010), elucidating the underlying genetic variations and the multiple interacting genes that confer increased susceptibility may improve our understanding of how PMDD symptoms develop.

Evidence for an interaction between the altered ESR2 in PMDD and catechol-o-methyltransferase (COMT) Val/Val genotype has been reported in a human haplotype analysis of 91 women with PMDD and 56 controls (Woods et al., 2000). COMT is an enzyme involved in multiple functions, such as estrogen metabolism (Hay et al., 1994) and has been hypothesized to tune prefrontal cortical activation through the regulation of dopamine levels (Belelli et al., 2006). The Val/Val genotype has been associated with decreased dopamine levels in the PFC and tuning efficiency (Soares, 2014). Thus, the authors speculate that a Val/Val genotype accompanied by an ESR2 variation might be a factor that could increase the susceptibility toward a dysphoric state via decreased PFC efficiency and disinhibited subcortical activity. Replication of this finding in a larger sample size, as well as the implementation of a neuroimaging protocol to explore the PFC-amygdala circuit in parallel with a detailed assessment of PMDD symptoms will be needed to further test this hypothesis.

While the magnitude of hormonal fluctuation does not seem significantly altered in women suffering from PMDD, an altered brain response to normal hormonal fluctuation could explain the changes in mood and behavior. Several lines of evidence support this concept: preliminary *in vivo* evidence from a small pilot (PET) study in five women with PMDD has shown that relative to healthy controls, women with PMDD experience a smaller change in 5-HT-receptor 1A binding throughout the menstrual cycle (Jovanovic et al., 2006). In animal models of hormonally induced depression via progesterone withdrawal, depression-like behavior can be modulated through specific serotonergic mechanisms or receptor subtypes respectively. Li and colleagues report that activation of 5-HT_{1A} receptors or inhibition of 5-HT_3 receptors rapidly decreases immobility in the forced swim test (FST), a prominent model for assessing antidepressant-like behavior in rodents. The FST differentiates between active (swimming and climbing) and passive (immobility) behavior when rodents are forced to swim in a cylinder with no escape options. Conversely, blocking 5-HT_{1A} receptors, activating 5-HT_3 receptors, or 5-HT_7 receptors increased depression-like behavior in rats in the FST (Li et al., 2013).

The 5-HTT mediates the recapture of serotonin from the synaptic cleft back into the cell. It is the therapeutic target of the currently most widely prescribed class of antidepressants: the SSRI. SSRIs have been found to be more effective in treating premenstrual symptoms than other non-SSRI drugs or a placebo (Dimmock et al., 2000; Shah et al., 2008). It is of particular interest that the pattern of response to drug therapy is different in patients with PMDD compared to patients suffering from a MDE. PMDD patients respond within the first menstrual cycle to SSRI-treatment (Halbreich and Kahn, 2003), suggesting that an imbalance in the serotonergic system may be of particular relevance to the development of PMDD symptoms.

In the context of serotonergic alternations in PMDD, BDNF has also been implicated. Depression has been shown to be associated with decreased BDNF expression, which can be reversed by antidepressant treatment (Lopez et al., 2013). In a study by Oral and colleagues, PMDD women are associated with increased BDNF levels and increased heat-shock protein 70 (HSP70) levels in the luteal phase compared with controls (Oral et al., 2013). These findings seem contrary to the previous findings by Lopez et al. However, Oral et al. discuss the possibility that increased HSP70 levels, as a molecular defense mediator against proteotoxic stress, might reflect cellular distress in PMDD women and that the respectively increased BDNF levels could be a compensatory mechanism potentially leading to resolved PMDD symptoms in the follicular phase. These compensatory mechanisms seem to fail in depressed patients. Findings of a recent study suggest a relationship between a specific BDNF polymorphism (BDNF Val66Met) and impaired fronto-cingulate cortex activation in response to an emotion processing task displaying angry or fearful emotions in the luteal phase of PMDD women (Comasco et al., 2014). As this interaction just appears to be present in the luteal phase, Comasco and colleagues suggest declining progesterone levels to trigger this phenomenon and discuss these changing progesterone levels to act via direct or chloride pump-mediated influence of BDNF on the GABAergic system. Furthermore, the BDNF Met allele lowers the sensitivity to 5-HT signaling (Martinowich and Lu, 2008), which may influence antidepressant efficacy in PMDD women (Comasco et al., 2014). Martinowich and Lu hypothesize that an increase in extracellular 5-HT, for instance after SSRI use, might increase BDNF levels because inhibition of 5-HTT facilitates serotonergic transmission through 5-$HT_{4,6,7}$ receptor subtypes (Martinowich and Lu, 2008).

Progesterone withdrawal associated with allopregnanolone increase in the luteal phase of the menstrual cycle has been hypothesized to be implicated in PMDD (Backstrom et al., 2011, 2014). Allopregnanolone is known for its similarities with benzodiazepines (Majewska et al., 1986), which can cause drowsiness, poor concentration, and memory impairment (Holbrook et al., 2000). Therefore, heightened allopregnanolone levels have been hypothesized to exhibit similar effects in the brain (Backstrom et al., 2014). Contrary to this hypothesis, a study by Girdler and colleagues found lower luteal phase allopregnanolone levels in PMDD patients with higher anxiety and irritability scores (Girdler et al., 2001). Furthermore, greater luteal phase allopregnanolone concentrations have been shown to be associated with improved symptom ratings in PMDD patients (Wang et al., 1996). A possible explanation for these unexpected findings has been proposed by Backstrom and colleagues: PMDD symptom severity seems to be related to allopregnanolone serum concentration in an inverted U-shaped

curve (Backstrom et al., 2011). Negative mood symptoms occur when the serum concentration of allopregnanolone is similar to endogenous luteal phase levels, while low and high concentrations have less effect on mood (Backstrom et al., 2014). This recent hypothesis is extended by the suggestion that negative mood symptoms in women with PMDD could be caused by an increased $GABA_A$ receptor sensitivity to allopregnanolone (Backstrom et al., 2014). Allopregnanolone levels have also been reported to increase in the brain after acute and chronic treatment with SSRIs (Lovick, 2013), providing evidence for a direct or indirect connection of allopregnanolone with the serotonergic system. The mechanism by which SSRIs increases allopregnanolone levels is thought to involve direct stimulation of 3α-hydroxysteroid dehydrogenase (3α-HSD), an important enzyme in the allopregnanolone biosynthesis (Compagnone and Mellon, 2000). Not only the susceptibility of the GABAergic system toward allopregnanolone seems to be altered in PMDD, GABA levels might also be abnormal. For instance, Epperson and colleagues found a reduction in the cortical GABA levels during the follicular phase in those with PMDD compared with healthy controls (Epperson et al., 2002). In healthy women, cortical GABA levels fluctuate across the menstrual cycle with decreasing levels from the follicular phase to the luteal phase, whereas the opposite occurred in PMDD women (Epperson et al., 2002).

CONCLUSIONS AND PERSPECTIVES

Pharmacological and behavioral approaches have been combined to demonstrate the critical role of sex steroid hormones in mediating effects on synaptic plasticity, memory, mood, and cognition. Several studies have taken advantage of available selective pharmacological tools and knockout mice to elucidate the underlying molecular mechanism of the observed behavioral and electrophysiological effects. These underlying mechanisms are complicated. Many of them involve rapid non-genomic action on presynaptic receptors like D1 receptors, NMDA receptors, and $GABA_A$ receptors. Furthermore, sex hormones act on multiple levels, simultaneously, as well as the interacting neurotransmitter systems that are largely interwoven.

Depending on the neurotransmitter system, sex hormone can exhibit facilitative, excitatory or suppressive, inhibitory effects on neurotransmission. For instance, progesterone has been shown to suppress the excitatory glutamate response (Hausmann and Gunturkun, 2000) and facilitates GABAergic neurotransmission through its action at $GABA_A$ receptors (van Wingen et al., 2008), while estrogen exhibits facilitating effects on glutamate transmission (Smith and Woolley, 2004) and suppresses GABA inhibitory inputs. The promoting effect of estrogen on glutamatergic neurotransmission, especially at NMDA receptors (Gazzaley et al., 1996; Woolley et al., 1997; Adams et al., 2004), is the inciting factor for synaptic plasticity and subsequently learning and memory (Foy et al., 1999). Furthermore, estrogen is known to promote dopamine release in the striatum, which might be mediated by the inhibitory effect of estrogen on GABA release, as dopamine terminals are influenced by GABAergic inputs. Thus, a decrease in inhibitory tone might facilitate DA release. This interaction between excitation and inhibition modulated by sex hormones is a key factor for understanding how sex hormones impact neuronal activity in the brain. Estrogen may produce its mentioned effects on cognition and mood especially through modulation of serotonergic function (Epperson et al., 2012b). Estrogen can increase serotonin levels and decrease 5-HT reuptake (Koldzic-Zivanovic et al., 2004), which allows 5-HT to

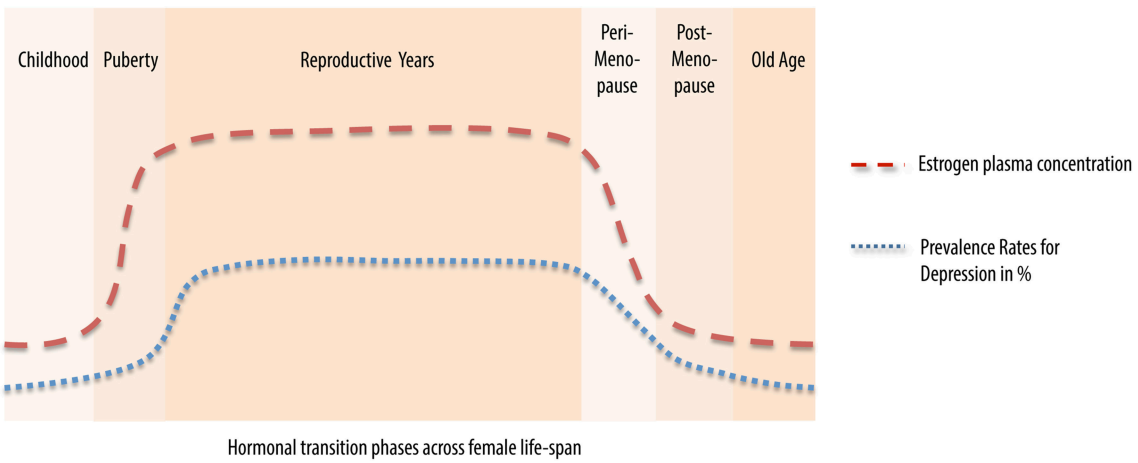

FIGURE 2 | Vulnerability for development of a depressive illness corresponds to main hormonal transitions across the female lifespan. During childhood (0–9 years), a phase associated with low estrogen plasma levels, the prevalence rate for depression ranges between 2 and 3% (Kashani et al., 1983; Lewinsohn et al., 1994). When estrogen levels start rising in puberty (10–15 years), so does the prevalence rate for depression, up to 8% (Angold et al., 1998). During reproductive years, a phase when estrogen and progesterone levels peak, prevalence rates vary between 21 and 38% (Kessler et al., 1993; Angold et al., 1998). Estrogen and progesterone levels start declining during perimenopause (41–51 years), drop considerably postmenopausally (45–65 years) and remain fairly stable during old age (above 65 years). This drop in sex steroid levels is paralleled by a decrease in prevalence rates for depression from 23 to 26% (Cohen et al., 2006a; Freeman et al., 2006; Unsal et al., 2011; Tamaria et al., 2013) during the hormonal transition phases to rates of 1–5% during old age (Tamaria et al., 2013).

remain longer in the synaptic cleft and exhibit prolonged effects on postsynaptic receptors.

Variations in hormone levels across the human lifespan exhibit pivotal actions in the human body and seem to heighten the risk of developing certain mood disorder and neurodegenerative pathologies. For women, hormonal transitions such as postpartum, menopause, and subtle fluctuations across the menstrual cycle seem to predispose women to mood disturbance (**Figure 2**), beginning with a heightened risk of developing a depressive episode following puberty (Soares and Zitek, 2008). Beyond the scope of postpartum or perimenopausal ovarian hormone loss, women can be also be more vulnerable on a monthly basis, across the menstrual cycle. Times of sex hormone withdrawal, as seen before onset of menses, are likely to predispose women to menstrual cycle related diseases such as PMDD.

To summarize, neurotransmitter systems do not work in isolation and sex hormones act on multiple sites, highly intertwined with serotonin, dopamine, GABA and glutamate. Fluctuating hormone levels across the human lifespan may be particularly significant for the etiology of neuropsychiatric diseases that display a prominent sexual dimorphism, such as Alzheimer's disease and depression. A better understanding of the underlying mechanisms that focus on activation of sex steroid specific pathways that could contribute to individual vulnerability to such diseases might allow for more effective treatment and prevention in the future.

Promising approaches include the targeting of estrogen receptors in a tissue-specific way using selective estrogen receptor modulators (Gambacciani, 2013; Mirkin et al., 2014). Further studies are on how treatment responses differ between different hormonal states. Promising future strategies involve the integration of basic with clinical neuroscience research, drawing resources from postmortem work, and innovative animal models for sex hormone associated affective disorders, such as the mouse model for PPD (Galea et al., 2008) to inform human *in vivo* neuroimaging experiments in health and disease. Such human neuroimaging studies could benefit from the application of the recently developed MR-PET hybrid scanners that allows for a combination of fMRI and PET, and could also accommodate real-time pharmacological interventions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2015.00037/abstract

Table S1 | Overview of genomic and non-genomic signaling properties for estrogen and progesterone. Here, we limit the overview for estrogen-signaling to estradiol (E2), the predominant estrogen during reproductive years for estrogen activity (Weis et al., 2008). This table provides a basic summary of the key features for common genomic and non-genomic estradiol/progesterone signaling, a more detailed review can be found elsewhere (Leonhardt et al., 2003; O'Lone et al., 2004; Marino et al., 2006; Singh et al., 2013).

Table S2 | General classification of main neurotransmitter systems (dopamine, serotonin, GABA, glutamate).

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Fetal hormonal programming of sex differences in depression: linking women's mental health with sex differences in the brain across the lifespan

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Women's health has traditionally been thought of in the realm of reproductive health, and that includes women's mental health (i.e., perinatal psychiatry). However, we now know there are significant sex differences in many chronic diseases, including brain disorders. Thus, understanding the causes of sex differences in disorders of the brain, within and outside of reproduction, is critical to understanding women's mental health and healthcare needs. In order to accomplish this, it is necessary for neuroscience to adopt a "sex-dependent" and/or "sex-specific" lens on investigations of the brain. In this review, we make the case for depression, which has among the largest sex differences in disorders of the brain.

Major depressive disorder (MDD) recently became the number one cause of disability worldwide (Murray and Lopez, 1997; Ustun et al., 2004; World Health Organization, 2012). Importantly, the incidence of MDD in women is twice that of men (Kessler, 2003; Kendler et al., 2006), and thus understanding its pathophysiology has widespread implications for attenuation and prevention of disease burden, particularly in women. Over 40 years of research implicate hormonal dysregulation underlying mood disorders (Board et al., 1956; Gibbons and McHugh, 1962; Coplan et al., 2000; Brouwer et al., 2005; Kurt et al., 2007; Barim et al., 2009), particularly involvement of hypothalamic-pituitary-adrenal (HPA) and HP-gonadal (HPG) axes (Board et al., 1956; Gibbons and McHugh, 1962; Plotsky et al., 1998; Young and Korszun, 2002; Swaab et al., 2005). Central dysregulation of hormonal axes can precede MDD onset suggesting a role for hormonal abnormalities in female MDD vulnerability. Ours and others' work demonstrated that the vulnerability for sex-dependent risk for MDD begins in *fetal* development (McClellan et al., 2010; Goldstein et al., 2011; Zuloaga et al., 2012a,b; Carbone and Handa, 2013; Seney et al., 2013). Despite these findings, a number of confounds (state vs. trait, treatment, age, and recurrence) present challenges to elucidating the contribution of hormonal or genetic sex (Seney et al., 2013) to the co-occurrence of hormonal dysregulation and mood disorders.

HPA AXIS IMPLICATED IN MDD

A central role for the HPA axis in MDD was initially expressed clinically. Depressive symptoms/MDD co-occurred with endogenously elevated cortisol (Sonino et al., 1998) or exogenously administered corticosteroids (Kelly et al., 1980; Ling et al., 1981). Studies demonstrated elevated levels of cortisol in plasma, CSF, and 24-h urine samples, high CSF corticotrophin releasing hormone (CRH) levels, blunted responses to CRH administration, and non-suppression of cortisol secretion on the dexamethasone suppression test in MDD (Carroll et al., 1976, 1981; Jarrett et al., 1983; Nemeroff et al., 1984; Halbreich et al., 1985; Holsboer et al., 1985; Banki et al., 1987; Evans and Nemeroff, 1987; Rubin et al., 1987; Heim et al., 2001; Newport et al., 2003; Raison and Miller, 2003). HPA axis dysregulation was related to age (Nelson et al., 1984a,b; Bremmer et al., 2007), depression subtype (Brouwer et al., 2005), recurrence (Poor et al., 2004), and treatment response, albeit inconsistently (Nemeroff et al., 1991; De Bellis et al., 1993; Veith et al., 1993; McKay and Zakzanis, 2010). One potential confound was whether HPA axis dysregulation reflected clinical state or diagnostic trait. A meta-analysis of >1500 individuals (Vreeburg et al., 2009), demonstrated that hypercortisolemia, present in currently depressed individuals (Trestman et al., 1993; Ahrens et al., 2008), persisted after recovery (Vreeburg et al., 2009), while other studies reported abnormal blunted cortisol response to stress in recurrent cases (Ahrens et al., 2008). In either case, findings suggested HPA dysregulation as a trait. In contrast, some studies showed resolution of hypercortisolemia with treatment (Vythilingam et al., 2004; Lok et al., 2012), arguing that HPA dysregulation was due to clinical state. Elevated baseline

cortisol, enhanced CRH sensitivity, and lack of responsivity to dexamethasone suppression also predicted relapse vulnerability and sustained remission (Zobel et al., 1999; Appelhof et al., 2006; Ising et al., 2007). Despite this evidence, HPA-axis targeted treatments are not reliably effective in MDD, although show some success as anti-depressant adjuncts (Jahn et al., 2004) or improvement of cognitive deficits (Young et al., 2004).

Despite substantial data supporting sex differences in HPA functioning during stress in healthy populations (Kudielka and Kirschbaum, 2005; Goldstein et al., 2010) and MDD women (Holsen et al., 2011, 2013), reports of sex differences in the HPA axis and MDD are inconsistent. Men, but not women, with MDD demonstrated increased ACTH pulsatility (Young et al., 2007a) and elevated cortisol compared with non-depressed men and women (Bremmer et al., 2007; Hinkelmann et al., 2012). However, depressed women vs. men (Poor et al., 2004) and non-depressed women (Young and Altemus, 2004; Chopra et al., 2009) also expressed hypercortisolemia. Study inconsistencies may be related to timing of cortisol assessments or may reflect methodological confounds, such as age of study subjects (e.g., post-menopausal women differ from premenopausal women and thus sex differences differ), chronicity of illness (e.g., sustained illness may produce blunted cortisol response rather than hypercortisolemia), or low statistical power to detect sex differences which may vary in effect size, depending on characteristics of the sample (details next paragraph). Further, genetic background likely affects HPA axis dysregulation, as demonstrated in studies showing increased ACTH and cortisol in males (but not females) homozygotic for the *alpha*(2)-adrenoreceptor gene and females (but not males) homozygotic for the *beta*(2)-adrenoreceptor gene (Haefner et al., 2008). Collectively, these findings offer initial evidence of sex differences in the role of HPA axis in MDD pathophysiology and emphasize the importance of considering genetic variation in HPA axis-associated genes.

Some studies report no effect of sex on HPA axis deficits in MDD (Carroll et al., 1976; Nelson et al., 1984b; Dahl et al., 1989; Maes et al., 1994; Deuschle et al., 1998; Brouwer et al., 2005; Vreeburg et al., 2009), although some of these studies were not designed initially to investigate sex differences, introducing potential confounds, such as: oversampling women (thus small samples of men) and low statistical power to test for sex differences (Brouwer et al., 2005; Young et al., 2007a; Vreeburg et al., 2009; Hinkelmann et al., 2012); lack of control for use of oral contraceptives or estrogen-replacement therapy (Brouwer et al., 2005) affecting plasma cortisol levels (Kirschbaum et al., 1999); and disregard for menstrual cycle phase or menopausal status during data collection. These confounds present significant challenges to understanding study inconsistencies on sex differences in HPA-MDD associations and their implications for women's mental health.

HPG AXIS IMPLICATED IN MDD

Post-puberty adolescence is a key period during which sex differences in MDD begin to emerge, initially during ages 13–15, with the largest increase in late adolescence (e.g., Hankin et al., 1998). However, few studies have focused on understanding why the higher rate of MDD in girls than boys is initiated during this period. This is unfortunate since puberty is an important critical period for brain plasticity likely arising from differential flooding of the brain with gonadal hormones (Schulz et al., 2009), and further sexual differentiation of the brain as the prefrontal cortex fully develops during ages 18–22 years. Evidence for HPG axis-MDD associations also came from studies of polycystic ovarian syndrome (Himelein and Thatcher, 2006) and literature relating women's reproductive biology to mood fluctuations and depression (Steiner, 1992; Bloch et al., 2000; Payne, 2003; Angold and Costello, 2006; Young et al., 2007b; Graziottin and Serafini, 2009; Brummelte and Galea, 2010). Although there has been less examination of HPG deficits in MDD in men, lower testosterone has been reported (Schweiger et al., 1999; Seidman et al., 2001). HPG dysregulation in MDD has included androgens (Baischer et al., 1995; Rubinow and Schmidt, 1996; Schweiger et al., 1999; Seidman et al., 2001; Weiner et al., 2004), estrogens (Young et al., 2000), and pituitary function (Daly et al., 2003). Women with persistent MDD had two times the risk of earlier perimenopausal transition, higher FSH, and lower estradiol levels, suggesting an early decline in ovarian function (Young et al., 2000; Harlow et al., 2003). Further, depressive symptom severity was associated with low estradiol levels (Baischer et al., 1995).

HPA-HPG INTERACTIONS IMPLICATED IN MDD

Dysregulation of HPA and HPG axes interact in MDD. Low levels of estradiol with unopposed progesterone in premenopausal MDD was associated with decreased inhibitory feedback on HPA function during stress, resulting in elevated cortisol in MDD compared to healthy women or men (Young and Altemus, 2004). Transient dysregulation of HPA axis during the luteal menstrual phase was reported in premenstrual syndrome (Rabin et al., 1990; Roca et al., 2003). Further, using functional MRI, our group showed *hypo*activity in stress-responsive regions in *premenopausal* MDD women was significantly associated with decreased estradiol and increased progesterone levels during the late follicular menstrual phase (Holsen et al., 2011). In *perimenopausal* MDD women, these brain regions were associated with *hyper*cortisolemia and *hyper*activity (Holsen et al., 2013). These imaging studies suggest a complex interplay between HPA and HPG axes, dependent on age and cycle timing. From a brain circuitry point of view, MDD involves hypothalamic (HYPO) nuclei (paraventricular and ventromedial), central amygdala (AMYG), hippocampus (HIPP), anterior cingulate, medial and orbital prefrontal cortices (ACC, mPFC, OFC) (Dougherty and Rauch, 1997; Mayberg, 1997; Drevets et al., 2002; Sheline et al., 2002; Rauch et al., 2003), regions dense in glucocorticoid and sex steroid hormone receptors (MacLusky et al., 1987; Clark et al., 1988; Handa et al., 1994; Kawata, 1995; Tobet and Hanna, 1997; Donahue et al., 2000; Östlund et al., 2003). These regions develop in sex-dependent ways, in part driven by gonadal hormones. There is now a substantial body of functional imaging work relating regulation of mood with endocrine

function, e.g., Goldstein et al., 2005, 2010; Protopopescu et al., 2005; Amin et al., 2006; Stark et al., 2006; Dreher et al., 2007; Wang et al., 2007; Pruessner et al., 2008; van Wingen et al., 2008a,b, 2009; Root et al., 2009; Andreano and Cahill, 2010.

SHARED MOOD AND ENDOCRINE CIRCUITRY ARE SEXUALLY DIMORPHIC

In vivo imaging and postmortem studies demonstrated sex differences in brain volumes (or nuclei) of regions associated with MDD, although there is little work focused on sexual dimorphisms in MDD *per se*. *In healthy women compared with men*, relative to cerebrum size, findings supported greater relative volumes of HIPP (Filipek et al., 1994; Giedd et al., 1996; Murphy et al., 1996; Goldstein et al., 2001), ACC (Paus et al., 1996; Goldstein et al., 2001), and OFC (Goldstein et al., 2001). *In men*, there are relatively greater volumes of AMYG (Giedd et al., 1996; Goldstein et al., 2001), HYPO (Swaab and Fliers, 1985; Allen et al., 1989; Goldstein et al., 2001), and paracingulate gyrus (Goldstein et al., 2001; Paus et al., 1996). Recently, a number of new studies have emerged further characterizing sex-dependent circuitry (Ruigrok et al., 2013), connectivity (Ingalhalikar et al., 2014), and potential mechanisms (Raznahan et al., 2010; Kang et al., 2011; Goldstein et al., 2013; Lenz et al., 2013; Nguyen et al., 2013). Developmental pathways involve, in part, gonadal hormone regulation, seen in model animal (McEwen, 1983; Simerly et al., 1990; Tobet et al., 1993, 2009; O'Keefe et al., 1995; Park et al., 1996; Tobet and Hanna, 1997; Gorski, 2000; Chung et al., 2006) and human (Goldstein et al., 2001; Raznahan et al., 2010) development. In fact, preclinical studies demonstrated lasting effects of prenatal adverse events on HPA axis and noradrenergic stress systems (Takahashi et al., 1992; Weinstock et al., 1992; Vallee et al., 1997; Weinstock, 1997). These included hypothalamic and hippocampal structure and function (Takahashi et al., 1992; Matsumoto and Arai, 1997; Weinstock, 1997), with effects that occurred through programming a "hyperactive" system more vulnerable to adult depressive and anxiety-like behaviors and autonomic nervous system deficits, among others (Weinstock et al., 1992; Henry et al., 1994; Barker, 1995; Seckl, 2001; Majdic and Tobet, 2011; Zuloaga et al., 2011; Carbone et al., 2012). Analogous to timing of these events in animals, mid-to-late gestation in humans is a particularly vulnerable time for the impact of prenatal events on *sex-dependent* brain development (Tobet et al., 2009; Majdic and Tobet, 2011; Zuloaga et al., 2011; Carbone et al., 2012), and recent preclinical and clinical studies implicated earlier gestation (Mueller and Bale, 2008; Howerton et al., 2013).

Preclinical studies also demonstrated sex differences (greater in females than males) in a number of domains, including: (1) greater placental glucocorticoid transfer (Montano et al., 1993; Fameli et al., 1994); (2) greater immobility in tasks associated with MDD phenotypic behavior (Alonso et al., 2000); (3) increased ACTH, corticosterone, and glucocorticoid receptor binding (Weinstock et al., 1992; McCormick et al., 1995; Regan et al., 2004); (4) increased corticosterone sensitivity (Rhodes and Rubin, 1999); (5) greater susceptibility to changes following loss of $GABA_B$ receptor function (McClellan et al., 2010; Stratton et al., 2011); (6) greater susceptibility to cell death in AMYG following developmental exposure to dexamethasone (Zuloaga et al., 2011); and (8) greater susceptibility to diet-induced hepatosteatosis and insulin growth factor-1 deficits (Carbone et al., 2012). In humans, at the level of the brain, there have been fewer studies of sex differences in MDD, although some reported decreased HIPP and increased AMYG volumes, greater in females than males (Vakili et al., 2000; Janssen et al., 2004; Weniger et al., 2006). Collectively, preclinical studies support the hypothesis that prenatal exposures (particularly those implicating stress circuitry pathways) facilitate altered programming of stress-related endocrine and neural circuits implicated in the sex-dependent development of depressive-like behavior. Although parallel studies in humans are still in their infancy, we and others are currently testing the hypothesis that *prenatal maternal* disruption of stress-immune pathways will, in the context of genetic background, result in vulnerability for the sex-dependent risk for MDD in the offspring (Handa et al., 1994; Majdic and Tobet, 2011).

CONCLUSIONS

The number one cause of disability worldwide is MDD, and women are two times the risk of men. This represents ∼350 million people worldwide, approximately 16 million in the U.S. alone (WHO October 2012 Fact Sheet). Depression is comorbid with many chronic diseases that are also associated sex differences in risk (Goldstein et al., 2011, 2013). Thus, depression is a major public health problem with substantial economic, social and disease burden that, we argue, requires a sex-dependent lens to understand its pathophysiology. There are key naturalistic opportunities for the study of this higher risk in women, and that is when the brain is differentially flooded with or depleted of gonadal hormones, i.e., fetal development, puberty, pregnancy, and perimenopausal-menopause transition. The evidence briefly discussed here supports the hypothesis that the etiology of sex differences in MDD begins in fetal development and emerges post-puberty. Its onset can be catalyzed by pregnancy (postpartum depression) and the menopausal transition (when there is an increase in MDD onset). The fact that these particular periods during the lifespan have significant implications for MDD onset is consistent with an important role for steroid hormones in MDD. This underscores the importance of promoting further inquiry into the development of adjunctive neuroendocrine treatments, dependent on timing across the lifespan. This lifespan approach to studying sex differences in disorders, like depression, also illustrates how maternal health (e.g., pregnancy), women's mental health, and sex differences in disorders of the brain are linked. Thus, we have argued the importance for preclinical and clinical neuroscience to incorporate a sex-dependent and/or sex-specific lens on investigations ranging from the cellular-molecular level to circuitry, systems, and behavior, an argument that recently was underscored by the new directive from NIH to incorporate this perspective in designs of preclinical studies (Clayton and Collins, 2014). We believe this will provide the basis for the development of sex-dependent therapeutics which will enhance progress to greater efficacy.

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Influence of sex and stress exposure across the lifespan on endophenotypes of depression: focus on behavior, glucocorticoids, and hippocampus

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Sex differences exist in vulnerability, symptoms, and treatment of many neuropsychiatric disorders. In this review, we discuss both preclinical and clinical research that investigates how sex influences depression endophenotypes at the behavioral, neuroendocrine, and neural levels across the lifespan. Chronic exposure to stress is a risk factor for depression and we discuss how stress during the prenatal, postnatal, and adolescent periods differentially affects males and females depending on the method of stress and metric examined. Given that the integrity of the hippocampus is compromised in depression, we specifically focus on sex differences in how hippocampal plasticity is affected by stress and depression across the lifespan. In addition, we examine how female physiology predisposes depression in adulthood, specifically in postpartum and perimenopausal periods. Finally, we discuss the underrepresentation of women in both preclinical and clinical research and how this limits our understanding of sex differences in vulnerability, presentation, and treatment of depression.

Keywords: sex differences, depression, HPA axis, hippocampal neurogenesis, adolescence

INTRODUCTION

SEX DIFFERENCES IN DEPRESSION

There are a number of sex differences in incidence, manifestation, symptoms, and treatment efficacy of neuropsychiatric disorders, however often these sex differences are ignored in the literature (Cahill, 2006). Even at the cellular levels, chromosomal influences (XX or XY genotype) can have profound effects on the cellular activity of every cell in the body, including neurons (Penaloza et al., 2009; Straface et al., 2012). Thus, it is curious that such a fundamental aspect of cellular function and physiology is largely ignored when understanding the neural basis and treatment of diseases (**Box 1**).

Epidemiological findings consistently show a sex disparity in the lifetime prevalence of depression, with women being twice more likely to be affected (Gutierrez-Lobos et al., 2002). This sex difference in prevalence is seen across cultures (Seedat et al., 2009), emerges during adolescence (Nolen-Hoeksema and Girgus, 1994), and is most apparent during the reproductive years (i.e., 25–50 years; Gutierrez-Lobos et al., 2002). Indeed, there is an increased incidence of depression in women during periods associated with dramatic fluctuations in gonadal hormones particularly during the postpartum and perimenopausal periods (Hendrick et al., 1998; Cohen et al., 2006). Alternatively, when the perinatal period is disturbed, males may be more vulnerable than females to develop other neuropsychiatric diseases, such as autism and schizophrenia, across the lifespan (Stevenson et al., 2000; Kent et al., 2012). Several biological and psychosocial theories have been put forth to explain the underlying cause of the sex differences in depression, but the most prominent neurobiological hypothesis emphasizes the role of gonadal hormones (Hammarstrom et al., 2009). Sex differences in depression extend beyond prevalence rates and course of illness as the symptom profile and clinical presentation differs between men and women. Several studies report that women are more likely to present with comorbid anxiety disorders (Sloan and Kornstein, 2003; Keers and Aitchison, 2010) as well as atypical depression, which is associated with hypersomnia, hyperphagia, or excessive fatigue (Young et al., 1990; Silverstein, 2002). Interestingly, sex is also implicated in antidepressant efficacy, with selective serotonin reuptake inhibitors (SSRIs) being more effective in alleviating symptoms in women, and tricyclic antidepressants (TCAs) being more effective in alleviating symptoms in men (Keers and Aitchison, 2010). However, sex differences in antidepressant efficacy are not always seen (Parker et al., 2003; Dalla et al., 2010). As we discuss later in this review, animal studies of depression have predominantly used male subjects, and thus our understanding of what underlies this differential antidepressant efficacy is limited. The available animal literature on sex-dependent antidepressant efficacy reveals mixed findings (Dalla et al., 2010); for example, some studies suggests that SSRIs are more efficacious in alleviating depressive-like behavior in female rats (Gomez et al., 2014), but others show a higher efficacy in males (Lifschytz et al., 2006). However, the latter study did not account for estrous cycle phase, which may affect depressive-like behavior and antidepressant efficacy. Together these data point to a critical role of sex and gonadal hormones on depression risk, manifestation, and treatment.

Box 1 | A call to action: the use of sex as a factor in research.

Preclinical studies on depression are essential to our understanding of the disease mechanisms, and in the discovery and screening of novel therapeutics. Despite the higher prevalence of depression in women, and the sex differences in the disease symptomology and pathophysiology, animal models of depression continue to be predominantly carried in male animals, and continue to overlook the role of sex hormones. The "default" use of male animals can be partly attributed to the reluctance of researchers to account for the variability associated with the fluctuation in female hormones (Beery and Zucker, 2011; Zucker and Beery, 2010). Neglecting sex differences and the role of female sex hormones in animal models of depression may be one of the reasons for the poor translation of findings from preclinical to clinical research (Belzung, 2014). The recent move by the National Institute of Health to insist that researchers use both males and females in preclinical studies is a step in the right direction (Clayton and Collins, 2014). The lack of investigation into the influence of sex, however, is not limited to preclinical research. The inclusion of both males and females in clinical trials, as per legislative requirements or recommendations (Merkatz et al., 1993), is not sufficient. There should be a move toward ensuring sufficient male and female sample sizes, and toward analyzing data from clinical trials by sex; i.e., sex should be analyzed as a variable and not merely a covariate to obtain statistically meaningful information on sex differences in depression and antidepressant treatment. Such practices will surely inform our search for new antidepressant treatments that are efficacious and safe in both males and females.

Stress and HPA involvement in depression

Exposure to chronic stress is tightly linked to the development of depression (reviewed in Tennant, 2002). The hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine stress system (reviewed in Ulrich-Lai and Herman, 2009) exhibits a number of changes in at least a subpopulation of depressed patients, with key features being elevated basal cortisol levels, disrupted diurnal cortisol secretion patterns, and HPA negative feedback dysregulation (Parker et al., 2003; Ising et al., 2007; Schule, 2007; Stetler and Miller, 2011). The HPA negative feedback system can be tested with the administration of dexamethasone, a synthetic glucocorticoid that suppresses cortisol secretion in healthy but not depressed individuals (Carroll et al., 1968; Ising et al., 2007). Chronic treatment with antidepressants can restore the negative feedback function of the HPA axis that either slightly precedes or is coincident with the alleviation of depressive symptoms (Ising et al., 2007). Interestingly, antidepressant effects to normalize HPA negative feedback dysregulation are more tied to remission in women than in men (Binder et al., 2009). Many animal models of depression emphasize the role of stress (reviewed in Yan et al., 2010), and HPA axis dysregulation is used as a measure of a depressive-like endophenotype in such models (Christiansen et al., 2012). Different types of stressors can profoundly influence the effects on depressive phenotypes with generally unpredictable psychological stressors more likely to promote depressive-like behaviors than predictable stressors, which can sometimes provide resilience to depressive-like behaviors (reviewed in McEwen, 2000, 2002; Parihar et al., 2011; Suo et al., 2013). Furthermore, greater allostatic load is associated with more profound effects on depression and the hippocampus (reviewed in McEwen, 2000, 2002). More profound HPA axis dysregulation as a result of chronic stress is seen in female rats when compared to males, marked by larger elevations in corticosterone (CORT), the main glucocorticoid in rodents (Dalla et al., 2005). These findings indicate that HPA dysregulation is seen in both humans and rodents, and this effect may be more profound in females. Moreover, females have naturally higher levels of CORT than males (reviewed in Viau, 2002) and this may contribute to higher incidence of depression in females. It should be noted that other stress hormones such as corticotropin releasing hormone and adrenocorticotropic hormone have been implicated in depression but are beyond the scope of this review and are reviewed elsewhere (e.g., Valentino et al., 2012).

The hippocampus and depression

The hippocampus is a highly plastic structure that is sensitive to the effects of stress and sex hormones, both of which are closely linked to depression. A meta-analysis confirmed that untreated depression is associated with a smaller volume of the hippocampus in depressed patients that have been depressed for at least 2 years (McKinnon et al., 2009). The smaller hippocampus associated with depression is an effect that is more prominent in men than women (Frodl et al., 2002) and in middle-aged and older patients (McKinnon et al., 2009). Furthermore, chronic antidepressant exposure appears to increase hippocampal volume in treatment-responding women more so than men (Vakili et al., 2000; reviewed in Lorenzetti et al., 2009). The hippocampus is a highly plastic structure in adulthood (reviewed in Leuner and Gould, 2010), and hippocampal volume fluctuations may be due to changes in neurogenesis, neuropil, and/or apoptosis. Postmortem studies reveal decreased cell proliferation in the dentate gyrus of the hippocampus of depressed patients (Boldrini et al., 2012). Similarly, hippocampal neurogenesis is reduced in every animal model of depression examined so far (Jaako-Movits et al., 2006; Green and Galea, 2008; Bessa et al., 2009; Brummelte and Galea, 2010a). Chronic but not acute antidepressant treatment restores the depression-model induced decrease in neurogenesis (Green and Galea, 2008; Bessa et al., 2009). Intriguingly, depressed women taking antidepressants have a larger ratio of immature to mature neurons in the hippocampus (an increase in neurogenesis) compared to controls, but the same relationship was not seen in men (Epp et al., 2013). These findings are consistent with the findings that women taking antidepressants show increased hippocampal volume compared to men (Vakili et al., 2000). Furthermore, the effect of antidepressants to induce neurogenesis in the hippocampus is not seen in older depressed patients (Lucassen et al., 2010; Epp et al., 2013), which may be consistent with the lack of efficacy of antidepressants to alleviate depression in older patients (Lenze et al., 2008). Neuropsychological evidence also suggests functional hippocampal impairment in depression, further supporting the role of the hippocampus in the pathophysiology of the disease. For

example, a meta-analysis of 726 patients with depression showed neurocognitive impairment, most severely in episodic, declarative memory (Zakzanis et al., 1998), a hippocampal-dependent memory system.

Animal models also show sex-dependent alterations in hippocampal plasticity as a result of chronic stress or chronic corticosterone treatment. Interestingly, chronic footshock stress reduced newly produced cells in the dentate gyrus of individually-housed young adult male rats, but increased new cells in young adult female rats (Westenbroek et al., 2004). Chronic restraint stress reduces neuropil (branch points and dendritic length) in the apical dendrites of CA3 pyramidal cells of the hippocampus in male rats but in the basal dendrites of CA3 pyramidal cells in female rats (Galea et al., 1997). Chronic CORT treatment reduced the density of immature neurons in the dorsal and ventral hippocampus of young adult male rats, but only the ventral hippocampus in young adult female rats (Brummelte and Galea, 2010b). While some researchers suggest that females are less susceptible to the damaging effects of chronic stress than males, this is very much dependent on the nature, duration of the stressor and the background hormonal environment of the female, with generally higher levels of ovarian hormones contributing to fewer damaging effects of stress (Shors et al., 2001; Conrad et al., 2012). Nonetheless these findings suggest that more research is needed to examine how stress alters plasticity in the hippocampus, how these changes in neuroplasticity translate into behavior and disease susceptibility, and how ovarian hormones contribute to this process. Further, stress and ovarian hormones can certainly influence risk for depression and plasticity of other limbic areas, such as the prefrontal cortex, and neurotransmitters, such as serotonin and dopamine, but this is beyond the scope of this review and are addressed elsewhere (e.g., Valentino et al., 2012; Goldstein et al., 2014).

Examining depressive-like endophenotypes in animal models

Anhedonia, i.e., the loss of pleasure or interest in previously pleasurable experiences, is one of the core symptoms of clinical depression. Not surprisingly, many animal models of depression focus on modeling anhedonia as a central behavioral endophenotype, typically by measuring the consumption of or preference for sucrose solutions. While reductions in the hedonic value of sucrose as a result of chronic unpredictable stress is seen in both male and female rats, the effect is more profound in male rats (Grippo et al., 2005; Dalla et al., 2005, 2008; Kamper et al., 2009). Because sucrose consumption/preference tests were first developed in male rodents, and because baseline sucrose consumption in females may fluctuate with the estrous cycle (Clarke and Ossenkopp, 1998), it may not be an ideal model of anhedonia in female rodents. On the other hand, other stress-induced depressive-like behaviors are more evident in female rats. For example, female rats show more despair-like behavior (immobility) on the forced swim test following chronic mild stress (Sachs et al., 2014) but not following chronic CORT treatment (Kalynchuk et al., 2004), an effect that may be related to sex differences in basal CORT levels (reviewed in Viau, 2002). As mentioned earlier, there are also reported sex differences in the symptoms of depression and in the prevalence of comorbid disorders; women are more likely to present with co-morbid anxiety and somatic complaints, whereas men are more likely to present with co-morbid alcohol and substance abuse (Marcus et al., 2008). Clearly, more clinical and preclinical research is needed to examine sex differences in the symptoms, and in the treatment alleviation of certain symptoms of depression.

The current article explores how adverse events present during developmental windows such as the prenatal period, the early postnatal period, and adolescence, are linked to increased vulnerability to neuropsychiatric disorders in adulthood. Many sex differences in the brain are programmed early in life (Paus, 2010). It perhaps is not surprising then that the sex differences associated with neuropsychiatric disorders also have a developmental component. The following sections will outline how prenatal, postnatal, adolescent or adult perturbations in stress or excessive stress hormones influence vulnerability to develop depression in both humans and animal models with a special emphasis on the behavioral analysis, HPA axis modulation, and neuroplasticity in the hippocampus.

PRENATAL MANIPULATIONS AND VULNERABILITY TO DEPRESSION

Prenatal stress limits fetal growth and gestational length (reviewed in Seckl and Holmes, 2007), and is consequently associated with an increased risk for depression, anxiety, schizophrenia, and more recently autism (reviewed in Bale et al., 2010; Baron-Cohen et al., 2014). During gestation, the placental enzyme 11β-hydroxysteroid dehydrogenase type II inactivates excessive levels of maternal or exogenous cortisol to protect the fetus from high cortisol. However, there are sex differences in the response of this placental enzyme to stress exposure which could contribute to which sex is more vulnerable to excessive maternal stress (reviewed in Clifton, 2010). For example, the female, but not male, placenta exhibits an increase in 11β-hydroxysteroid dehydrogenase type II expression in response to prenatal exposure to betamethasone (a synthetic glucocorticoid) prior to preterm delivery (Stark et al., 2009). Although seemingly contradictory to the general pattern that females are more vulnerable to depression than males, this finding is consistent with findings that males are more vulnerable to neurodevelopmental disorders that can be triggered during gestation, such as autism, and poor outcome after preterm birth (Stevenson et al., 2000; Kent et al., 2012). Thus, the developmental timing of stress exposure may play a pivotal role in which sex is more likely to develop depression and the reader is directed to excellent reviews on the subject (Andersen, 2003; Teicher et al., 2003; Brenhouse and Andersen, 2011). Further research investigating how sex differences in placental function mediate developmental outcome will provide a better understanding of why males are more vulnerable to the effects of prenatal stress than females.

Prenatal stress affects depressive behavior

Gestational stress can contribute to the development of mood disorders in the mother during pregnancy (Lancaster et al., 2010). Depression during pregnancy in the mother increased emotional responses in infant boys, but not girls (Gerardin et al., 2011), and increased risk for depression in adolescence (Pawlby et al., 2009; Pearson et al., 2013). Furthermore, maternal anxiety during

pregnancy is associated with a greater risk for depressive symptoms in adolescent girls (Van Den Bergh et al., 2008) and a greater risk for attention deficits in young and adolescent boys (Van Den Bergh et al., 2006; Loomans et al., 2011). Together, these studies suggest that maternal mood during gestation may differentially affect behavioral outcome in boys and girls.

Animal models of depression that induce prenatal stress have been used to examine how early life perturbations differentially affect male and female offspring. Generally these animal models involve exposing the pregnant dam to predictable or unpredictable stress (reviewed in Weinstock, 2008). Despite the fact that clinical research points to strong associations between prenatal stress and likelihood to develop depression, preclinical research has yielded mixed results. However, it should be noted that the third trimester equivalent in rodents is the first week postnatal and thus one reason why there may be differences between human and animal studies is timing of "trimester" in different species (Kleiber et al., 2014). Timing of stress onset plays a critical role in how it affects offspring depressive-like behavior. For example, one study found that prenatal stress increased depressive like behavior (increased immobility in the forced swim test) of adult male mice only when stress occurred during the first week of gestation but not during mid- or late-gestation (Mueller and Bale, 2008) which would be akin to the first vs. second trimester. However, others have shown that prenatal restraint stress during the last week of gestation (akin to late in the second trimester) increased depressive-like behavior (increased immobility in the forced swim test) in adult males and females, although in males it is only seen when restraint occurs three times per day during the last week of gestation (Alonso et al., 1991; Morley-Fletcher et al., 2003; Szymańska et al., 2009; Van Den Hove et al., 2014). Age of offspring at behavior testing is also critical as prenatal stress had the opposite effect on depressive-like behavior (decreased immobility in the forced swim test) in pre-pubertal (P33; Schroeder et al., 2013) and adolescent male and female rats (Rayen et al., 2011). Alternatively, direct administration of CORT to the dam during days 10–20 of gestation (equivalent to second trimester) increased depressive-like behavior (increased immobility in the forced swim test) for both adolescent male and female rats (Brummelte et al., 2012). Ultimately, duration of prenatal stress, frequency of stress exposure, timing of prenatal stress, type of stressor employed, species, and age of offspring at testing influence depressive-like behavior of the offspring (reviewed in Huizink et al., 2004; Weinstock, 2011). Additional research addressing how these differences in methodologies influence offspring outcome will be valuable for understanding how prenatal factors influence vulnerability to depression in males and females.

Prenatal stress affects development of HPA axis

Prenatal stress can have variable programming effects on the HPA axis of offspring depending on sex, the paradigm of prenatal stress and the part of the HPA axis (basal, stress peak, stress recovery) analyzed (Barbazanges et al., 1996; Zagron and Weinstock, 2006). For instance, prenatal stress for even 1 week of gestation can result in prolonged CORT recovery after acute stress in adult female but not in adult male rats (Weinstock et al., 1992; McCormick et al., 1995). However, after more intense prenatal stress (stress three times per day during last week of gestation and equivalent to second trimester) even male rats displayed prolonged CORT recovery after restraint (Maccari et al., 1995; Morley-Fletcher et al., 2003) and both sexes exhibit disrupted diurnal CORT rhythm (Koehl et al., 1999). Alternatively, social defeat stress during the last week of gestation (equivalent to second trimester) exaggerated the peak CORT response after restraint in both adult male and female rats (Brunton and Russell, 2010). These findings suggest that more intense paradigms of prenatal stress are capable of reprogramming the male HPA axis whereas females seem to be sensitive to milder forms of prenatal stress.

In clinical studies, prenatal stress has been associated with HPA axis dysregulation in infants, adolescents, and adults (reviewed in Glover et al., 2010). However, there are limited studies directly assessing how sex mediates the effects of prenatal stress on the development of the HPA axis. Both maternal prenatal anxiety and prenatal exposure to synthetic glucocorticoids (either dexamethasone or betamethasone) increased stress reactivity in girls only (De Bruijn et al., 2009; Alexander et al., 2012). However, whether HPA axis dysregulation persists in girls or whether it emerges later on in boys remains unknown. Future clinical studies directly analyzing how sex mediates the relationship between prenatal stress and development of HPA axis are necessary to address this gap.

Prenatal stress affects hippocampal plasticity

Prenatal stress persistently decreased cell proliferation in the hippocampus of juvenile, adolescent, adult, and aged male rodents (Lemaire et al., 2000; Mandyam et al., 2008; Rayen et al., 2011; Belnoue et al., 2013) and in pre-pubescent rhesus monkeys (2–2.5 years old; Coe et al., 2003). The prenatal stress-induced decrease in cell proliferation is more prominent in the ventral hippocampus (Zuena et al., 2008) and the ventral hippocampus is associated with stress and anxiety more so than the dorsal hippocampus (reviewed in Fanselow and Dong, 2010). In contrast, the effect of prenatal stress on hippocampal cell proliferation in female offspring is more complicated. Prenatal stress decreased hippocampal cell proliferation in adolescent female rats (Rayen et al., 2011) but had variable effects in adult females. Prenatal stress diminished cell proliferation in adult (5 months old) female rats (Mandyam et al., 2008) and in aged (2 year old) females (Koehl et al., 2009) but the later study did not see the same effect in younger adult female offspring. Although both studies employed the same prenatal stress paradigm, there were differences in onset of prenatal stress (gestational day 14 or 15) as well as breeding (shipped pregnant vs. breeding in house) that may explain this conflict (Laroche et al., 2009). Prenatal stress diminished number of immature neurons (doublecortin expressing) in adolescent male and female rats (Rayen et al., 2011) and decreased neurogenesis in adult male rodents (Lemaire et al., 2000; Belnoue et al., 2013). Prenatal stress affects other aspects of hippocampal morphology as well as other regions of limbic system and are reviewed elsewhere (e.g., Charil et al., 2010; Weinstock, 2011). In non-human primates, there are known effects of prenatal excessive glucocorticoids to show damage to different areas of the hippocampus (reduction in volume) into middle age (Uno et al.,

1994). In humans, one study found that prenatal stress diminished use of hippocampal-dependent strategies in a spatial task (Schwabe et al., 2012). However, whether these effects are paralleled in hippocampal volume, related to risk for depression, or differentially affected by sex remain unknown and likely need to be taken into account to fully understand the effects of prenatal and postnatal adversity on the hippocampus in humans (Frodl and O'Keane, 2013).

While there has been robust evidence from preclinical and clinical studies that prenatal stress negatively affects offspring outcome, prenatal stress can also diminish quality of maternal care (Champagne and Meaney, 2006) and even serve as a model of postpartum depression (PPD; Smith et al., 2004; Leuner et al., 2014). Thus, studies that employ prenatal stress paradigms essentially result in a mix of prenatal and postnatal adversity, obscuring the degree to which poor functional outcome can be attributed to *in utero* stress alone. Cross-fostering of prenatally stressed with non-stressed rodent pups reverses certain behavioral and endocrine effects (Barros et al., 2006; Del Cerro et al., 2010; Perez-Laso et al., 2013). In humans, studies have found that some of the negative effects of prenatal stress on child outcome are mediated by the early postnatal environment, such as presence of postpartum mood disorders or poor maternal care (Kaplan et al., 2008; Bergman et al., 2010; Rice et al., 2010). These studies highlight the prenatal and postnatal environments as potent mediators in offspring development.

POSTNATAL MANIPULATIONS AND VULNERABILITY TO DEPRESSION

Postnatal stress affects depressive behavior

Several forms of postnatal early life stress, such as sexual, physical, or emotional abuse as well as parental loss, neglect, or mental illness constitute increased risk for adult mood and anxiety disorders (Famularo et al., 1992; Pelcovitz et al., 1994; reviewed in Heim and Nemeroff, 2001). In fact, recent work suggests that depression is harder to remit if the individual has a history of early childhood adversity such as physical abuse (Fuller-Thomson et al., 2014). One of the most potent elements of early life stress is the quality of parental care (Ladd et al., 2000). Indeed, postpartum mood disorders such as PPD disrupt a healthy mother–infant bond and can have a profound effect on child development (reviewed in Goodman and Gotlib, 1999; Lovejoy et al., 2000; Ashman et al., 2002; Deave et al., 2008; Van Hasselt et al., 2012). For example, boys of PPD mothers have lower IQ (reviewed in Grace et al., 2003; Azak, 2012) while both adolescent girls and boys of PPD mothers are more likely to develop depression and anxiety (Pilowsky et al., 2006; Murray et al., 2011) as well as a propensity for violent behavior (Hay et al., 2003). Of course PPD is not often seen in isolation, and antenatal depression (as described above) or maternal depression can also influence emotional behaviors such as antisocial behavior in children (Kim-Cohen et al., 2005; Hay et al., 2010). Indeed maternal depression, which is often defined as depression within a year after giving birth, can critically affect cognitive and emotional development of children (reviewed in Goodman and Gotlib, 1999). The reader is directed to a review on maternal depression during gestation or postpartum differentially affecting child outcome (Hay et al., 2008). Thus, PPD alone or in conjunction with additional depressive episodes can profoundly influence child development and represents a risk for behavioral and cognitive disturbances.

Disruptions to the early postnatal environment have also been linked to depressive-like behavior in animal models of depression. One of the most common postnatal paradigms used is maternal separation in which pups are removed from the dam for 3 hours per day during the postnatal period (reviewed in Schmidt et al., 2011). As may be expected, offspring outcome after maternal separation differs in terms of the duration, timing and method of separation (dam or pups removed from home cage). Again it is worth noting that the first week of postnatal life in rodents is equivalent to the third trimester in humans. Maternal separation during the first two postnatal weeks (equivalent to third trimester) or the entire postnatal period (from birth to weaning) increased immobility in the forced swim test in adult male and female rats (Lee et al., 2007; Aisa et al., 2008; Lajud et al., 2012). Another interesting model of postnatal adversity is the limited bedding model in which dams are provided with limited bedding and nesting material during the early postnatal period (equivalent to third trimester). Denying the mother sufficient nesting material consequently decreased maternal behaviors and increased erratic and abusive maternal behaviors (Ivy et al., 2008) which result in increased immobility in the forced swim test in adolescent rat offspring (Raineki et al., 2012). Thus, substantial loss of proper maternal care during the postnatal period can impact both male and female vulnerability to depression and sensitivity to stress well beyond the time they are dependent on the mother.

Postnatal stress affects development of HPA axis

Maternal separation influences HPA axis development in a time and sex-specific manner. For instance, maternal separation during the first two postnatal weeks (equivalent to third trimester) reduced basal CORT in adult females but not in adult males (Slotten et al., 2006). However, maternal separation lasting the entire postnatal period elevated basal CORT in adult females (Aisa et al., 2008). Maternal separation during the first 2 weeks or entire postnatal period blunted the peak CORT response to stress in pre-weaning (Litvin et al., 2010) and adolescent male rats (Ogawa et al., 1994). However, by adulthood, maternal separation had no significant effect on CORT reactivity in either adult male rats or female rats (Plotsky and Meaney, 1993; Plotsky et al., 2005; Slotten et al., 2006). Interestingly, maternal separation during the first two postnatal weeks (equivalent to third trimester) resulted in exaggerated adrenocorticotropic hormone secretion after restraint (Liu et al., 2000) but not after exogenous corticotropin releasing hormone in male rats (Wigger and Neumann, 1999), suggesting that maternal separation may have a more potent effect on sensitivity of the pituitary gland to different stressors in male rats. Maternal separation in rodents closely approximates parental neglect in humans, which is also associated with disruptions to the developing HPA axis (reviewed in Tarullo and Gunnar, 2006). For example, toddlers raised in the extreme psychosocial neglect in Romanian orphanages exhibit blunted secretion of cortisol over the day (Carlson and Earls, 1997) but exhibit exaggerated cortisol secretion as young children (Gunnar et al., 2001). Similarly, PPD and early life abuse are associated with HPA axis hyperactivity in adolescents and adult

men and women, respectively (PPD: Halligan et al., 2004; abuse: Heim et al., 2002, 2008). However, timing of abuse onset is a critical factor as if the onset of abuse was early, then young girls present with hypocortisolemia whereas young boys present with hypercortsolemia (Doom et al., 2013). Together, these data suggest that the postnatal environment can either blunt or exaggerate HPA axis activity depending on sex, timing and type of postnatal stress.

Postnatal stress affects hippocampal plasticity

Maternal separation lasting the first two postnatal weeks (equivalent to third trimester) diminished cell proliferation, but not the survival of new neurons in the dentate gyrus, in adult male rats, which was reversed by adrenalectomy (Mirescu et al., 2004). Maternal deprivation for 24 h on postnatal day 3 enhanced number of immature neurons in male rats but diminished number of immature neurons in female rats at weaning (Oomen et al., 2009). However, by adulthood, the number of immature neurons and survival of new neurons was diminished in the ventral hippocampus of adult male rats but not in female rats (Oomen et al., 2010, 2011). This suggests that males experience more dynamic and long-lasting changes to neurogenesis than females in response to maternal separation. In humans, children exposed to maternal depression since birth exhibit no significant change in hippocampal volume (Lupien et al., 2011) whereas adult hippocampal volume is vulnerable to the effects of childhood maltreatment (Chaney et al., 2014). This may be partly explained by low socioeconomic status which is associated with reductions in hippocampal volume in children/adolescents (Noble et al., 2012) as well as increased risk for PPD (Goyal et al., 2010). There are also sex differences as another study found that self-reported low maternal bonding was associated with small hippocampal volume in women but not in men (Buss et al., 2007). Future research addressing how maternal care interacts with socioeconomic status to affect hippocampal volume will help shed light on how the hippocampus can be affected by maternal care and the postnatal environment throughout the lifespan.

While maternal separation has provided clear evidence that separation from the dam alters offspring development, there are caveats to this model. For instance, there are changes in maternal care that occur based on the separation and reuniting of the dam with her pups (Pryce et al., 2001; Macrí et al., 2004; Own and Patel, 2013). Moreover, maternal separation results in increased depressive like behavior in the dam (increased immobility in the forced swim test; Boccia et al., 2007). Our laboratory has developed a model of PPD in which dams are treated with high levels CORT (40 mg/kg/day) throughout the postpartum period which diminished maternal care and increased immobility in the forced swim test in the dam (Brummelte et al., 2006; Brummelte and Galea, 2010a). Because our model requires an injection to the dam, there is minimal separation (less than 1 min of separation each day) of the pups from their mother. Thus, our model more closely resembles the consistent levels of voluntary diminished maternal care or neglect typical of PPD as opposed to forced sessions of total maternal deprivation. We have shown that high maternal postpartum CORT increased anxiety-like behavior in adolescent male, but not female, rats (Brummelte et al., 2012). Furthermore, high maternal postpartum CORT diminished cell proliferation in the dentate gyrus of juvenile male but not female rats (Brummelte et al., 2006). CORT during pregnancy and the postpartum also increased peak CORT in response to restraint stress in both males and females (Brummelte et al., 2012). Altogether, these data suggest that high maternal CORT affects offspring HPA axis, hippocampal plasticity and anxiety-like behavior in a sex-specific manner, consistent with the effects of PPD on child development.

ADOLESCENCE MANIPULATIONS AND VULNERABILITY TO DEPRESSION

Adolescent stress affects depressive behavior

While the perinatal period has received much attention in terms of predisposing individuals to mood disorders, recent research has highlighted the importance of the adolescent period as schizophrenia, substance abuse, depression, and anxiety can emerge during adolescence (Costello et al., 2003; reviewed in Patton and Viner, 2007). It should be noted that adolescence broadly refers to the developmental window involving the transition from childhood to adulthood whereas puberty specifically refers to the maturation of the HPG axis and the subsequent gonadal hormone fluctuations occurring during adolescence (Spear, 2000). Stressful events during adolescence might have compounding effects on an already volatile state of emotional and social challenges during adolescence (Spear, 2000). Thus, stress exposure during the adolescent years could influence risk for depression as well as an increased vulnerability to neuropsychiatric disorders later in life (reviewed in McCormick and Mathews, 2007; Holder and Blaustein, 2014). Further, as indicated above, the consequences of perinatal stress may emerge during adolescence or even interact with adolescent stress to shape vulnerability to adult depression (Rueter et al., 1999; Goodyer, 2002). For instance, the presence of both antenatal depression and child maltreatment increased risk of depression by almost five-fold and increased risk of developing psychopathologies by twelve-fold in adolescents (Pawlby et al., 2011). Alternatively, positive family support can reduce the risk for depression in adolescent boys and girls depending on genetic background, supporting the idea that postnatal factors mediate risk for adolescent depression (Li et al., 2013).

The rise in gonadal hormones during puberty plays an important role in precipitating the sex differences in depression. In fact, as mentioned earlier the increased female prevalence of depression emerges during puberty (Ge et al., 1996). Interestingly, adolescent female rats spend more time immobile in the forced swim test than adolescent male rats even without any additional experimental manipulations (Leussis and Andersen, 2008). This suggests that animal models are also able to capture this early female vulnerability to depression. Stressors during adolescence can also differentially affect adolescent male and female depression-like behavior. Social isolation during early adolescence (P30-35) increased depressive-like behaviors (immobility in the forced swim test and increased latency to escape shock in the shuttlebox) in adolescent male, but not female, rats (Leussis and Andersen, 2008). Similarly, social defeat stress during early adolescence (P29-31; P35-44, respectively) increased immobility

in the forced swim test in adolescent male, but not female, rodents (Ver Hoeve et al., 2013; Iñiguez et al., 2014). However, when early adolescent rats are exposed to alternating episodes of social defeat and restraint, adolescent female, but not male, rats exhibited shorter latency to immobility in the forced swim test which persisted into adulthood (Bourke and Neigh, 2011). This suggests that perhaps adolescent males are more sensitive to social stressors but adolescent females are more sensitive to multimodal stressors. Others have found an opposite effect of adolescent social isolation only in females such as increased climbing behavior in the forced swim test (but not immobility) in adolescence and adulthood as well as increased sucrose preference in adulthood (Hong et al., 2012). Given that adolescence is characterized by a complex array of social behaviors and challenges, it is possible that there are sex differences in how adolescent animals are vulnerable to the effects of social stress. This may be an important point to consider in animal models assessing adolescent vulnerability to depression.

Adolescent stress affects HPA axis

The responsiveness of the HPA axis to stress during adolescence is different than that of the adult HPA axis (reviewed in Klein and Romeo, 2013). Additionally, the maturation of the HPA axis is dependent on the maturation of the HPG axis (Romeo, 2010) and the increased risk for depression during adolescence may be related to maturation of the HPA-HPG interactions (reviewed in Angold and Costello, 2006). After 5 days of restraint stress, CORT levels habituate in adolescent female, but not male, rats (Doremus-Fitzwater et al., 2009). Chronic social instability stress during adolescence exaggerates the peak CORT response after swim stress in adolescent male and female rats, but this effect is not seen in adulthood (Mathews et al., 2008). Chronic restraint stress throughout adolescence (P30-P52) significantly increased basal CORT levels for adult females, but not male, rats (Barha et al., 2011). However, 1 week of restraint during early adolescence (P26-33) blunted HPA axis reactivity in adult male rats but had no significant effect on adult female rats (Ariza Traslavina et al., 2014). Thus, hyperactivity of the HPA axis is observed in both males and females during adolescence, but timing of adolescent stress exposure results in sex differences in terms of the longitudinal effects on adult HPA axis. Early adolescent stress causes HPA hypoactivity in adult males (Ariza Traslavina et al., 2014) but stress throughout adolescence causes HPA hyperactivity in adult females (Barha et al., 2011).

Adolescent stress affects hippocampal plasticity

Chronic restraint stress throughout adolescence diminished neurogenesis (cell proliferation and survival) in the dentate gyrus in adult female rats, but slightly increased neurogenesis in adult male rats (Barha et al., 2011). Interestingly, this same pattern persists in social stress paradigms as social instability stress during adolescence diminished hippocampal cell proliferation in adolescent female rats (McCormick et al., 2010) while it enhanced cell proliferation and doublecortin-expressing cells in the dorsal hippocampus of adolescent male rats (McCormick et al., 2012). Moreover, social isolation during adolescence also diminishes cell proliferation specifically in the rostral hippocampus and immature neurons throughout the hippocampus in adolescent marmoset monkeys (Cinini et al., 2014). Unfortunately although while both male and female marmosets were used in the study, sex was not independently analyzed, so it is unclear whether sex differences were present in their findings to match those of previous studies in rodents (Cinini et al., 2014). Furthermore, chronic resident-intruder stress did not significantly affect hippocampal cell proliferation in adolescent male rats (Hanson et al., 2011). Together, these studies highlight the importance of stress and the adolescent period on hippocampal neurogenesis and that females are more likely to show reduced neurogenesis in response to adolescent stress compared to males. Both preclinical and clinical research has been investigating how stressors occurring during adolescence affect behavior and maturation of the brain. Future studies should also examine effects of adolescent stress on hippocampal volume and function both during adolescence and later in adulthood to help bridge the gap preclinical research with clinical research (reviewed in Andersen and Teicher, 2008; McCormick and Green, 2013).

ADULT MANIPULATIONS AND VULNERABILITY TO DEPRESSION

Depression in the postpartum

As discussed previously, PPD and other maternal mood disorders can affect child's risk for depression. Interestingly, the postpartum period is the time of greatest risk for women to develop depression (Drevets and Todd, 2005) and affects approximately 15% of women (Kornstein, 2002; Goodman, 2007; Wisner et al., 2013). To complicate matters, women are reluctant to take antidepressants during pregnancy or the postpartum with only 18% of depressed mothers seeking treatment (Marcus, 2009). During pregnancy and postpartum, levels of steroid hormones fluctuate dramatically which could contribute to the etiology of PPD (Hendrick et al., 1998; Bloch et al., 2003). Pregnancy and postpartum are characterized by sustained high flattened levels of glucocorticoids in both humans (Magiakou et al., 1996; Schule, 2007) and rodents (Lightman et al., 2001; Pawluski et al., 2009), which is a similar hormone profile observed in depressed patients (Stetler and Miller, 2011). Women who previously suffered from PPD reported more depressive symptoms and showed greater cortisol responses after exposure to a hormone-simulated pregnancy (Bloch et al., 2005), suggesting that in vulnerable women, the HPA axis and mood are altered in response to pregnancy hormones. An animal model of depression from our laboratory has shown that high levels of CORT directly administered to the dam increased depressive-like behavior (increased immobility in the forced swim test) and decreased dendritic complexity and cell proliferation in the hippocampus at the end of the postpartum period (Brummelte et al., 2006; Brummelte and Galea, 2010a; Workman et al., 2013). The ability of chronic antidepressants to increase neurogenesis in the hippocampus is tied to the liable nature of corticosterone (Huang and Herbert, 2006). Given the increased and flattened profile of cortisol during pregnancy and postpartum, it may not be surprising then that antidepressants prescribed at this time may not be as efficacious as they would be in cycling women. Indeed, there is little evidence to suggest that prescribed antidepressants work better than psychotherapy or placebo methods during the postpartum (reviewed in O'Hara and McCabe, 2013; De Crescenzo et al.,

Table 1 | Effects of stress exposure at different time points throughout life on depressive-like behavior (i.e., immobility in the forced swim test; FST) in preclinical research (first row) and on depression in clinical research (second row).

Vulnerability	Prenatal	Postnatal	Adolescent	Adult
♂ > ♀	↑ Immobility in FST • Variable stress, GD 1–7 (173) • Restraint 3×/day, GD 14–21 (172, 241, 254)	–	↑ Immobility in FST • Isolation (in adolescence; 141) • Social defeat (in adolescence, 115)	↓ Sucrose preference • Chronic mild stress (57, 120) ↑ Immobility in FST • Chronic corticosterone administration (119)
	–	–	–	–
♀ > ♂	↑ Immobility in FST • Restraint and suspension, GD 15–21 (254)	–	↑ Immobility in FST • No stress (in adolescence; 141) • Social defeat and restraint (in adolescence, 29)	↑ Immobility in FST • Chronic mild stress (213)
	↑ Depression • Antepartum anxiety (in adolescence, 253)	–	↑ Depression • No stress (84)	↑ Depression • No stress (98) • Perimenopause and postpartum (51, 109) ↑ Atypical depression • No stress (226, 273) ↑ Comorbid disorders • No stress (122, 228)
♀ = ♂	↑ Immobility in FST • Corticosterone administration, GD 10–20 (in adolescence; 34)	↑ Immobility in FST • 2–3 weeks of maternal separation (1-males not studied, 132, 135)	–	–
	↑ Depression • Antepartum depression (in adolescence; 193, 198)	↑ Depression • Postpartum depression (in adolescence; 174, 202) • Antepartum depression, abuse(194)	–	–

All data are based on outcome in adulthood unless otherwise specified. ♂ > ♀, Males more vulnerable than females; ♀ > ♂, females more vulnerable than males; ♀ = ♂, both sexes equally vulnerable; GD, gestation day.

2014). In addition to changes in HPA hormones across pregnancy and the postpartum, estradiol levels are elevated throughout the third trimester but drop dramatically after parturition, leading to the hypothesis that an "estradiol-withdrawal state" during the first few weeks after parturition contributes to PPD (Hendrick et al., 1998; Bloch et al., 2003). Our laboratory was first to show that withdrawal from a hormone-simulated pregnancy induced depressive-like symptomology (increased immobility in the forced swim test and sucrose anhedonia; Galea et al., 2001; Green et al., 2009) and reduced adult hippocampal neurogenesis in female rats (Green and Galea, 2008). There is also evidence that the postpartum period is associated with reduced plasticity in the hippocampus (Pawluski and Galea, 2006, 2007) and neuroplasticity is thought to be integral to antidepressant efficacy, as further discussed below (Wainwright and Galea, 2013).

Depression in perimenopause

The perimenopausal period is characterized by dramatic fluctuations in hormones followed by a persistent hypogonadal state (Burger et al., 2008). The period of transition into menopause lasts approximately 10 years and poses an increased risk to develop depression for women (Freeman et al., 2004; Cohen et al., 2006), suggesting a role of gonadal hormones in the etiology of the disease. Although a previous history of depression is a strong predictor of depression in the perimenopausal period (Freeman et al., 2004), the risk is also increased in women with no previous history of the disease (Cohen et al., 2006; Freeman et al., 2006). Antidepressant efficacy is also associated with the gonadal hormone status of postmenopausal women. In postmenopausal women with depression, SSRIs show better efficacy in those on hormone therapy (HT) in comparison with those not receiving HT (Thase et al., 2005; Pae et al., 2009; Kornstein et al., 2010), suggesting the efficacy of HT as an adjunct therapy alongside SSRI antidepressants in postmenopausal women. Similarly, the gonadal hormone status of men is implicated in depression, and correlational studies indicate that depressed men have lower levels of testosterone (Sachar et al., 1973; McIntyre et al., 2006). Interestingly, this effect seems to be more prominent in aging men, in

Table 2 | Effects of stress exposure at different time points throughout life on HPA axis in preclinical research (first row) and on depression in clinical research (second row).

Vulnerability	Prenatal	Postnatal	Adolescent	Adult
♂ > ♀	↑ Recovery • 3×/day, week GD 14–21 (152, 172)	↑ Peak CORT • Maternal separation 2–3 weeks (juvenile: 145; adolescent: 180)	↓ Peak CORT • Restraint, P26-33 (9)	–
	–	–	–	–
♀ > ♂	↑ Recovery • 1×/day, GD 15–19 (162) • 3×/week, GD 1–21 (264)	↓ Basal CORT • Maternal separation for 2 weeks (229) ↑ Basal CORT • Maternal separation for 3 weeks (males not studied; 1)	↓ Basal CORT • Restraint, P30-35 (in adolescence; 66) ↑ Basal CORT • Restraint, P30-52 (16)	↑ Basal CORT • Chronic mild stress (56)
	↑ Peak cortisol • Antepartum anxiety (61) • Antepartum glucocorticoids (2)	–	–	–
♀ = ♂	↑ Peak CORT • Social defeat, GD 16–20 (36)	–	↑ Peak CORT • Social instability stress, P30-45 (in adolescence; 158)	–
	–	↑ Basal CORT • Postpartum depression (99) • Abuse (106, 108)	–	–

All data are presented as outcome in adulthood unless otherwise specified. ♂ > ♀, Males more vulnerable than females; ♀ > ♂, females more vulnerable than males; ♀ = ♂, both sexes equally vulnerable; CORT, corticosterone; GD, gestation day; P, postnatal day.

which the age-related decline in testosterone may be related to depression (Carnahan and Perry, 2004). Moreover, testosterone as an adjunct therapy with antidepressants shows efficacy in alleviating depression symptoms in hypogonadal men (Seidman et al., 2001, 2009), a finding that was also supported by a meta-analysis (Zarrouf et al., 2009). Thus, the decline in gonadal hormones in aged men and women may predispose vulnerable individuals to depression and HT may be efficacious as an antidepressant adjunct therapy.

Depression in older age

With the aging of populations and the increasing life expectancy, interest in older-age depression has increased. Older-age depression often presents with comorbid medical conditions (Alexopoulos et al., 2002), is associated with poorer outcomes of comorbid illnesses (Sinyor et al., 1986; Palinkas et al., 1990; Michelson et al., 1996; Musselman et al., 1998) and higher rates of mortality (Rovner et al., 1991; Ganguli et al., 2002). Methodological differences may account for the wide range of reported prevalence rates of major depression in older individuals, which can be up to 9.4% in the community and 42% in nursing homes (reviewed in Djernes, 2006). In close to half of older individuals with depression, the illness takes on a chronic or relapsing course (Weyerer et al., 1995; Mojtabai and Olfson, 2004). Furthermore, the efficacy of antidepressants in this population is poor; this is evident in the high rates of treatment resistance in randomized control trials using first-line antidepressants, which are up to 81% with serotonin/norepinephrine reuptake inhibitors, and up to 77% with SSRIs (reviewed in Lenze et al., 2008). Treating older-age depression is further complicated by factors related to the higher comorbidity of medical illnesses, such as interactions with drugs frequently prescribed to older individuals (Spina and Scordo, 2002). Unfortunately, older-age depression is also underdiagnosed and/or under-treated (Steffens et al., 2000; Watson et al., 2003; Stek et al., 2004); this may be in part due to a unique symptom presentation in which older individual with depression are more likely to report somatic complaints rather than depressed mood (Small, 1991). Unfortunately there is very little information on whether sex differences are still seen in this population.

Major depression often presents in the context of dementia, with prevalence rates of about 17% in patients with Alzheimer's disease (Wragg and Jeste, 1989). Depression in the older population accelerates dementia and is associated with poorer cognitive outcomes (Bromberger et al., 2003). Interestingly, older individuals with mild cognitive impairment are more likely to develop dementia after diagnoses with major depression (Freeman et al., 2004; Schmidt et al., 2004). Moreover, evidence regarding the efficacy of antidepressant treatments in patients with dementia is weak; in a meta-analysis where only four randomized control trials met inclusion criteria, the authors suggest that there is no sufficient evidence to conclude that antidepressants are efficacious in individuals with depression and dementia (Bains et al., 2002). More recent meta-analyses also showed that neither response nor remission rates differ between antidepressant treatments and placebo in this population (Nelson and Devanand, 2011; Sepehry et al., 2012).

Table 3 | Effects of stress exposure at different time points throughout life on hippocampus in preclinical research (first row) and clinical research (second row).

Vulnerability	Prenatal	Postnatal	Adolescent	Adult
♂ > ♀	–	↓ Cell proliferation • Maternal separation 2 weeks (females not studied; 170) ↓ Immature neurons • Maternal deprivation, P3 (183, 184)	↑ Cell survival • Restraint, P30-52 (16) ↑ Cell proliferation, immature neurons • Social instability, P30-45 (in adolescence; 163)	↓ Cell survival • Chronic footstock stress (265) ↓ CA3 apical dendritic neuropil • Chronic restraint stress (80) ↓ Immature neurons (dorsal) • Chronic corticosterone (33)
	–	–	–	↓ Volume • At least 2 years of depression (77)
♀ > ♂	–	↓ Immature neurons • Maternal deprivation, P3 (at weaning; 182)	↓ Cell proliferation • Restraint, P30-52 (16) • Social instability, P30-45 (in adolescence; 161)	↑ Cell survival • Chronic footstock stress (265) ↓ CA3 basal dendritic neuropil • Chronic restraint stress (80)
	–	–	–	↑ Immature neurons • No stress, with antidepressant treatment (69) ↑ Volume • No stress, with Antidepressant treatment (249)
♂ = ♀	↓ cell proliferation, survival, immature neurons • Stress during week 3 of gestation, effects throughout lifespan (20, 128, 136, 155, 207, 279)	–	–	↓ Immature neurons (ventral) • Chronic corticosterone (33)
	–	↓ Volume • Maltreatment (44)	–	–

All data are based on outcome in adulthood unless otherwise specified. ♂ > ♀, Males more vulnerable than females; ♀ > ♂, females more vulnerable than males; ♀ = ♂, both sexes equally likely to develop depression. P, postnatal day.

With the continued growth of the older population, older-age depression should be regarded as a serious public health concern, and efforts to enhance its recognition and treatment are essential.

HPA function in older-age and its potential contribution to depression

Interestingly, there are marked changes in HPA function related to older-age. Cortisol levels increase dramatically (Van Cauter et al., 1996), and the normal fluctuations in diurnal cortisol rhythms are blunted with age (Ferrari et al., 2001). Furthermore, a meta-analysis showed that older-age is associated with HPA negative feedback dysregulation, which is significantly more prominent in women (Otte et al., 2005). Intriguingly, and as described earlier, such changes in HPA function mirror those seen in depressed patients regardless of age, and may therefore contribute to the increased risk of depression in older individuals. However, how sex differences in HPA function in this population may contribute to differences in depression risk or presentation is unclear and warrants further research.

Neuroplasticity in older-age may be implicated in antidepressant efficacy

While the neurobiology behind the lowered antidepressant efficacy in older individuals is not well understood, it may be explained by aging-related changes in neuroplasticity, particularly in the hippocampus. As mentioned above, a meta-analysis on hippocampal volume and depression found that a smaller volume of the hippocampus was associated with depression particularly in the older population (McKinnon et al., 2009). Further, many aspects of hippocampal plasticity are reduced in older-age, for example, there is a decline with age in neurogenesis levels (Couillard-Despres, 2013), synaptic proteins (Eastwood et al., 1994; VanGuilder et al., 2010), and spine densities (Anderson and Rutledge, 1996; Von Bohlen Und Halbach et al., 2006; Tsamis et al., 2010) in humans as well as rodents. Additionally, older-age is associated with reductions in serum brain-derived neurotrophic factor (Ziegenhorn et al., 2007), which is important for neural plasticity and compromised in depression (reviewed in Autry and Monteggia, 2012). The age-related reductions in gonadal hormones in both men (Ferrini and Barrett-Connor,

1998) and women (Burger et al., 2008) are, at least in part, responsible for the lowered malleability of the brain. Indeed, gonadal hormones are known to regulate neuroplasticity on many levels (Yang et al., 2004; Franklin and Perrot-Sinal, 2006; reviewed in Galea et al., 2013; Hamson et al., 2013). This reduced state of plasticity may render the brains of older individuals less malleable in response to antidepressant treatments. In fact, antidepressant-induced increase in neurogenesis is not seen in older depressed patients (Lucassen et al., 2010; Epp et al., 2013). This association between the lower state of plasticity and lower antidepressant efficacy aligns with newer theories of depression suggesting that alterations in neuroplasticity, beyond neurogenesis, may serve as a unifying mechanism through which antidepressant drugs produce their effect (reviewed in Wainwright and Galea, 2013). Therefore, if antidepressants work through neural remodeling, then antidepressant failure may result from an impaired ability of the nervous system to undergo change ("plasticity potential"). Evidently, preclinical and clinical research is warranted to explore this possibility.

Neuroplasticity and antidepressant efficacy during the postpartum

Curiously, low plasticity potential may also explain vulnerability to depression and/or reduced antidepressant efficacy in other populations, for example, women in the postpartum period. There is a shortage of randomized controlled trials investigating the effectiveness of antidepressants in the postpartum, however, the current evidence does not suggest that commonly prescribed antidepressant treatments are efficacious in this population (reviewed in O'Hara and McCabe, 2013; Sharma and Sommerdyk, 2013). Magnetic resonance imaging studies show a decrease in brain size and an increase in ventricular size during pregnancy, which persist in the postpartum period until 6 months post-delivery (Oatridge et al., 2002). The changes that occur in the maternal brain are poorly understood, and the mechanisms behind this decrease in size cannot be explained using imaging studies. Animal research supports the theory that early postpartum is associated with reduced plasticity at least in the hippocampus (Pawluski and Galea, 2006, 2007; Darnaudéry et al., 2007; Leuner et al., 2007; reviewed in Hillerer et al., 2014). Clearly, there are large changes in neuroplasticity associated with pregnancy and the postpartum, and a lower plasticity state may explain the reduced antidepressant efficacy by traditional antidepressants in this group. Thus, not only do we need to better understand how depression is different between women and men, but also how different periods within a woman's life can impact the development and treatment of depression.

CONCLUSIONS

The evidence we review here, with a focus on depression, suggests that sex interacts with developmental window and age to modulate the outcomes of stress exposure. Several facets of depression are differentially impacted by the interaction of sex and age, including vulnerability, symptomology, treatment efficacy and pathophysiology, as indicated by differences in HPA function and hippocampal plasticity. We highlight that males and females are differentially vulnerable to different types of stress across the lifespan, with males being more vulnerable to perinatal perturbations, females being more vulnerable later in life during peripartum, and both sexes being affected in adolescence and aging. These findings are summarized in **Tables 1–3**. Thus, in order to best understand depression and improve its treatment, researchers must not only utilize both sexes, but should acknowledge that evaluation and treatment in one sex may not be optimal for the other sex (for further discussion, please see **Box 1**). Moreover, given that pregnancy and the postpartum are accompanied by dramatic physiological changes, it should not be surprising that depression and antidepressant efficacy are different in these women compared to cycling women. Finally, we highlight the need for an improved understanding of depression and its treatment in older-age. We provide evidence suggesting that the lowered state of neuroplasticity in the older brain may contribute to the disease etiology and/or lowered antidepressant efficacy in this population. To conclude, we advocate for the inclusion of more female subjects, and for the analysis of results by sex in both preclinical and clinical research, for it is vitally important to begin to do systematically, as only in this way will we start to understand the powerful effects of sex on stress and depression risk.

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Functional and molecular neuroimaging of menopause and hormone replacement therapy

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The level of gonadal hormones to which the female brain is exposed considerably changes across the menopausal transition, which in turn, is likely to be of great relevance for neurodegenerative diseases and psychiatric disorders. However, the neurobiological consequences of these hormone fluctuations and of hormone replacement therapy in the menopause have only begun to be understood. The present review summarizes the findings of thirty-five studies of human brain function, including functional magnetic resonance imaging, positron and single-photon computed emission tomography studies, in peri- and postmenopausal women treated with estrogen, or estrogen-progestagen replacement therapy. Seven studies using gonadotropin-releasing hormone agonist intervention as a model of hormonal withdrawal are also included. Cognitive paradigms are employed by the majority of studies evaluating the effect of unopposed estrogen or estrogen-progestagen treatment on peri- and postmenopausal women's brain. In randomized-controlled trials, estrogen treatment enhances activation of fronto-cingulate regions during cognitive functioning, though in many cases no difference in cognitive performance was present. Progestagens seems to counteract the effects of estrogens. Findings on cognitive functioning during acute ovarian hormone withdrawal suggest a decrease in activation of the left inferior frontal gyrus, thus essentially corroborating the findings in postmenopausal women. Studies of the cholinergic and serotonergic systems indicate these systems as biological mediators of hormonal influences on the brain. More, hormonal replacement appears to increase cerebral blood flow in several cortical regions. On the other hand, studies on emotion processing in postmenopausal women are lacking. These results call for well-powered randomized-controlled multi-modal prospective neuroimaging studies as well as investigation on the related molecular mechanisms of effects of menopausal hormonal variations on the brain.

Keywords: estrogens, fMRI, HRT, hormones, menopause, neuroimaging, PET, SPECT

BACKGROUND

The change in ovarian hormone levels to which the female brain is exposed during the menopausal transition is likely relevant for neurodegenerative diseases as well as psychiatric disorders. However, the effects of the peri- and postmenopausal estradiol level decline and hormone replacement therapy (HRT) on the brain have only begun to be understood (Mueller et al., 2014). Undeniably, a substantial lack of agreement on the beneficial vs. detrimental effect of HRT on mental health, and concerns about potential adverse effects exist (Henderson and Greicius, 2010). Thus, the prevention and clinical management of mental health problems during the menopausal transition suffers from uncertainty, which renders research in this field of outmost importance.

During the last two decades menopause researchers have opted for an intermediate phenotype-based approach to further the psychobiological dissection of menopausal hormonal transition and HRT effects on mental health. Indeed, intermediate phenotypes (e.g., biochemical, endocrine, anatomical, physiological) represent quantifiable basic biological or behavioral processes, which are expected to be closer to the genetic underpinning as well as to the psychological functions that might be affected (Meyer-Lindenberg and Weinberger, 2006; Rasetti and Weinberger, 2011). Neuroimaging techniques are certainly suitable tools for investigating potential intermediate phenotypes related to the neurobiological correlates of menopause and HRT.

The present review summarizes the findings of studies of human brain function (i.e., functional magnetic resonance imaging (fMRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT/SPET), and magnetic resonance spectroscopy (MRS), of perimenopausal and postmenopausal women treated with unopposed estrogen or combined estrogen-progestagen replacement therapy. Additionally, studies of gonadotropin-releasing hormone (GnRH) agonist

treatment, as a pharmacological model of menopause, are also considered.

STATE-OF-THE-ART

To date, 35 functional neuroimaging studies have investigated brain function in peri- and postmenopausal women, and seven additional studies have used GnRH agonist intervention as a model of menopause (**Tables 1–6**). However, all studies are statistically under-powered since they include small samples, and they are also characterized by high heterogeneity in terms of study design. Differences regarding the study designs include: controlled randomization vs. cross-sectional trials, parallel vs. cross-over design, baseline vs. placebo control state, duration of the HRT use, unopposed estradiol vs. combined estrogen-progestagen therapy, route of administration and posology/dose of HRT, time between menopause and start of HRT ("critical window"), and chronological and reproductive age of the women. Dissimilarities regarding the neuroimaging data regard: neuroimaging techniques, neuropsychological paradigms within a cognitive domain, and statistical approaches for neuroimaging data processing and analysis. All together, these factors impeded any meta-analytical statistics.

COGNITIVE FUNCTIONING

The great majority of studies evaluating the effect of unopposed estrogen or combined estrogen-progestagen treatment on the menopausal and postmenopausal brain used cognitive paradigms (**Tables 1**, **2**). Longitudinal, randomized controlled trial studies of the effects of estrogen treatment—three to twelve weeks in duration—indicated a higher activation of fronto-cingulate regions during cognitive functioning (**Table 1**), as assessed by fMRI using verbal, non-verbal and spatial working memory tasks. However, in spite of higher activation in fronto-cingulate regions, no differences in working memory performance were noted (Shaywitz et al., 1999; Joffe et al., 2006; Dumas et al., 2010). One sample of healthy postmenopausal women with an average of 7 years of prior estrogen use, and randomly assigned to a placebo or three-month hormonal treatment, showed an estradiol, memory load-dependent, effect on frontal activation, with a greater modulatory effect during the more difficult visual verbal working memory task (Dumas et al., 2010). During a verbal memory task, inferior frontal and posterior parietal regions, and during a spatial working memory task, frontal, posterior cingulate, and parietal regions, activation was greater in peri- and postmenopausal women receiving a three-week transdermal estradiol treatment compared to placebo (Joffe et al., 2006). Besides an increased and decreased inferior frontal lobule activation during storage of verbal and non-verbal material, respectively, a relatively large, randomized, double-blind, placebo-controlled, cross-over trial of estrogen treatment (for three weeks) in perimenopausal women indicated also a sharpened hemisphere encoding retrieval asymmetry (HERA) effect, with higher left-frontal hemisphere activation during encoding, and higher right-frontal hemisphere activation during retrieval in a working memory task (Shaywitz et al., 1999).

Moreover, two randomized controlled fMRI trials (**Table 1**), with cross-over designs, including ten healthy postmenopausal women each, suggested a counteractive effect of progestagens on memory, with increased, but to a lesser extent, prefrontal cortex activation after four weeks of combined estrogen-progestagen treatment vs. placebo (Smith et al., 2006; Persad et al., 2009). On the contrary, one parallel study of postmenopausal women did not observe any effect of six months of combined hormonal treatment in comparison with placebo on verbal fluency and mental rotation (Davison et al., 2013).

Results of cross-sectional neuroimaging studies on cognitive function are sparse (**Table 2**), and interpretation is limited by sample selection and study set-up discrepancies (Gleason et al., 2006; Berent-Spillson et al., 2010, 2012; Maki et al., 2011). Combined estrogen- or estrogen-progestagen treatment was associated with increased bilateral activation of the medial temporal lobe during encoding in postmenopausal women (Gleason et al., 2006). Activation in the medial temporal lobe during verbal and figural memory tasks differed between postmenopausal women who started HRT before menopause and never users, with a more pronounced decrease in parahippocampal gyrus and increase in left hippocampus activation in the HRT users during verbal recognition (Maki et al., 2011). Furthermore, HRT was associated with a better verbal memory performance, indicating a beneficial effect of HRT (Maki et al., 2011). A relatively large study found generalized increased brain activation upon a visual working memory task in women who had used estrogen, with or without progestagen addition, for more than 10 years, and begun HRT within two years of the menopause, compared to non-users (Berent-Spillson et al., 2010). In the same sample, activation of the medial temporal lobes correlated positively with performance during a visual working memory task (Berent-Spillson et al., 2010). Moreover, differences between pre-, peri-, and postmenopausal women during a verbal, but not visual, memory task were found, independently of age, in frontal, prefrontal, and temporal cortices, with brain region activation being negatively correlated with estrogen levels (Berent-Spillson et al., 2012).

Finally, Maki and Resnick's longitudinal PET study provided evidence that estrogen treatment, with or without progestagen addition, modulates longitudinal changes in blood flow in brain regions implicated in cognitive function. Firstly, HRT users and non-users performing a verbal memory and a visual memory task showed, in a complex fashion, differences in the activation of regions subserving memory, such as the frontal lobe (Resnick et al., 1998). A smaller and overlapping sample was investigated two years later using the same study set-up. Greater activation of the temporal gyrus, hippocampus and insula regions, and the parahippocampal gyrus and inferior frontal gyrus, was observed during verbal and visual memory tasks, respectively, in long-term HRT users (more than two years) compared to non-users, thus indicating altered blood flow in brain areas subservicing memory in the presence of HRT (Maki and Resnick, 2000). However, in non-users other regions were also activated during the figural memory task compared to users (Maki and Resnick, 2000). Moreover, HRT was associated with better memory performance in the neuropsychological tests at both time points (Resnick et al., 1998; Maki and Resnick, 2000).

Notably, different components of the cognitive tasks contribute to the complexity of the many neuropsychological

Table 1 | Randomized controlled trial longitudinal studies neuroimaging cognitive functioning during HRT.

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique	Test	Comparison	Results	Notes
E								
Dumas et al., 2010	20 [S+2]	60 ± 6 60 ± 5	gr1 ($n = 10$): E(3m) gr2 ($n = 10$): placebo (3m) (pastET, $n = 6$)	fMRI (2x)	Visual verbal working memory	Placebo vs. ET	ET: [3 Back] ↑ miFG (BA 8), sFG l (BA 10+8), iFG r (BA 47), miTG r (BA 21), CG l (BA 24), precG l (BA 4), postcG l (BA 4), pCun r (BA 31); ET: [2 Back] ↑ mFG r (BA 8), iFG r (BA 47), miFG r (BA 8), postcG l (BA 4); NT: [1 Back] ↑ Ins r (BA 13), precG l (BA 6); ET: [0 Back] ↑ miTG r (BA 21), sTG r (BA 41)	= Accuracy, but variable bias in performance
Joffe et al., 2006	11 [−2≤S≤+1C]	51 ± 3 51 ± 4	PeriMP gr1 ($n = 5$): baseline + transdermal E(12w) gr2 ($n = 6$): baseline + placebo (12w)	fMRI (2x)	Verbal working memory	Placebo vs. ET	ET: ↑ mFL, FG (BA11), PCL/FL (BA5), pcG/PL(BA1), PL, Cau r, Thal r	= Performance, but HT: less errors of perseveration in verbal recall
					Spatial working memory	Placebo vs. ET	ET: [5–3 item] ↑ CG (BA32), sFG (BA9), Cun, pC (BA29)	
Shaywitz et al., 1999	46 [S:+1A]	51 ± 5 (33–61)	Pre- to postMP gr1: CEE (3w) + wash-out (2w) and placebo (3w) gr2: placebo (3w) + wash-out (2w) and CEE (3w)	fMRI (2x)	Verbal and non-verbal working memory	Placebo vs. ET	ET: [storage, verbal memory] ↑iPL, sFG [retrieval] ↑sFG r ET: HERA effect: ↑ l vs. r during encoding, ↑ r vs. l during retrieval	= Performance
E+P								
Davison et al., 2013	13 [S:≥+1A]	53 (50–54) 53 (50–55)	PostMP gr1 ($n = 6$): baseline + E P (6m) gr2 ($n = 7$): baseline + placebo (6m)	fMRI (2x)	Verbal fluency Mental rotation	Baseline vs. HT placebo vs. HT	n.s	= Performance
Persad et al., 2009	10 [S:≥+1C]	60 ± 1 (56–60)	PostMP gr1: E P (4w) + wash-out (4w) and placebo (4w) gr2: placebo (4w) + wash-out (4w) and E P (4w)	fMRI (2x)	Verbal memory	Placebo vs. HT	HT: ↑ pFC l (BA6+9), daC/mFC (BA24+32), pC (BA6), PC l (BA40+39)	= Performance
Smith et al., 2006	10 [S:≥+1C]	57 ± 1 (50–60)	PostMP gr1: E P (4w) + wash-out (4w) and placebo (4w) gr2: placebo (4w) + wash-out (4w) and E P (4w)	fMRI (2x)	Visual working memory	Placebo vs. HT	HT: ↑ pFC (BA44+45)	= Performance

See Appendix for acronyms/abbreviations.

Table 2 | Cross-sectional studies neuroimaging cognitive functioning during HRT.

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique / Tracer	Test	Comparison	Results	Notes
Berent-Spillson et al., 2012	56 [S:−3 S:−2 S:+1]	(42–61) 47 ± 3 53 ± 3 54 ± 3	(No HT ≥3m): gr1 ($n = 20$): PreMP gr2 ($n = 15$): periMP gr3 ($n = 32$): PostMP	fMRI	Episodic verbal memory (encoding and storage)	PreMP vs. periMP vs. postMP	PostMP: ↑ iFC r, PFC l, TP l periMP: ↑iFC l corr −: E and iFC l, TP l, PHC l, PC l	Different executive function, and verbal fluency, after adj for age
					Visual working memory		n.s.	
Dumas et al., 2012b	24 [S:+2]	59 ± 6 60 ± 5	gr1 ($n = 12$): E (3m) + antimuscarinic / antinicotinic / placebo gr2 ($n = 12$): placebo (3m) + antimuscarinic / antinicotinic /placebo	fMRI (2x)	Visual verbal working memory + cholinergic antagonist	ET vs. placebo	[Antimuscarinic]: E: ↓ mFG l (BA10), aCG r (BA24), iPL l (BA40), Ins r (BA13), sTG l (BA22) [Antinicotinic]: E: ↑ pCun r (BA31), ↑ paracentralL (BA5), ↓ PHG r (BA34)	= Performance
Maki et al., 2011	25 [S:+2]	60 ± 3 60 ± 3	(HT before final menstruation): gr1 ($n = 13$): E/CEE (P, n =?) gr2 ($n = 12$): NT	fMRI	Verbal memory	HT vs. NT	[recognition]: NT: ↑ PHG (BA35, 36), HT: ↑HC l [match]: HT: ↑ HC l [encoding-match]: NT: ↑ HC l	HT: ↑ verbal memory performance
					Figural memory	HT vs. NT	[recognition]: HT: ↑ PHG l (BA35, 27), perirhinal [recognition-match]: HT: ↑ PHG r (BA35)	
Berent-Spillson et al., 2010	55 [S:+2]	68 ± 6 64 ± 5 66 ± 5	PostMP (within 2y of MP and past HT≥10y): gr1 ($n = 17$): CEE gr2 ($n = 20$): CEE+P gr3 ($n = 18$): NT	fMRI	Visual working memory	ET+HT vs. NT	ET+HT: ↑ iFL r, aC, aIns l, PL r, sPL l, HC l, PHC, pC, raphe	= Performance Corr +: performance and activation Hipp r, meTL
						ET vs. NT	ET: ↑ sFL, aIns l, pIns, sPL r, iPL, HC l, PC	
						HT vs. NT	HT: ↑ pFC r, iFL r, sFL l, Pu l, Ins r, aIns l, pIns l, sPL r, HC, PHG l, pC, midbrain raphe	
						HT vs. ET	HT: ↑ sPC l, PHG ET: ↑ sFL r, pFC r, sPC r	
Gleason et al., 2006	23 [S:+2]	58 ± 5	PostMP gr1 ($n = 4$): opposed E gr2 ($n = 10$): opposed CEE gr3 ($n = 9$): NT	fMRI	Cognitive	HT vs. NT	HT: ↑ HC r, vpTL l	E > NT > CEE memory performance

(Continued)

Table 2 | Continued

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique / Tracer	Test	Comparison	Results	Notes
Maki and Resnick, 2000	28 [S:+2]	66 ± 6 68 ± 6	gr1 (n = 12): E (+P, n = 6) (pastET≈15y) +2y gr2 (n = 16): NT (pastET, n = 5) +2y	PET (2x) [^{15}O] water	Verbal memory	ET vs. NT Verbal vs. rest Over time	ET: ↑ HC r, Ins r, sTG l NT: ↑ ACC l	HT: ↑ memory performance
					Figural memory	ET vs. NT Figural vs. rest Over time	ET: ↑ pPHG r, iFG r NT: ↑ miTG r, ↓Cb r	
Resnick et al., 1998	32 [S:+2]	68 ± 6 65 ± 6	gr1 (n = 15): ET (+P, n = 7) gr2 (n = 17): NT (pastET, n = 5)	PET [^{15}O] water	Resting state	/	/	HT: ↑ figural and verbal memory
					Verbal memory	ET vs. NT Verbal vs. rest	ET: ↓ PHG r, PCun r, dFG r NT: ↑ iFC r, ↓ Hy l	
					Figural memory	ET vs. NT Figural vs. rest	ET: ↑ iPL r, ↓ PHG r, NT: ↑ visual ass cortex l, ↓ aThal l, MB r	

See Appendix for acronyms/abbreviations.

functions examined (**Tables 1**, **2**). Functional domains of memory, such as executive function, attention, visuospatial processing, and language, are subserved by different brain circuits, which may have variable sensitivity to specific interventions (Gibbs, 2010). Memory tasks have been used predominantly to assess menopausal and HRT effects on cognitive functioning. Working memory, mainly subserved by the dorsal prefrontal cortex in humans, is a limited-capacity storage system to maintain and manipulate information over short time periods and is critical for many daily activities (Gibbs, 2010). The visual-verbal N-back test of working memory, used by Dumas et al. (2010), allows the examination of different working memory loads (i.e., 0-, 1-, 2-, and 3- back conditions), from a condition of minimal memory load (0-) to one where subjects need to hold four letters in memory and update working memory as the next letter appears, alternatively seen as a cognitive stress model. Other verbal memory tasks, used by Shaywitz et al. (1999), Joffe et al. (2006), Persad et al. (2009), Berent-Spillson et al. (2012), Davison et al. (2013), involve learning and recall of word lists. Verbal memory has been suggested to be the most sensitive cognitive domain to the effect of HRT, and also a candidate proxy for dementia (Maki, 2013). The obvious discrepancy between imaging and cognition test results might be due to compensatory or deficient mechanisms, but also to differences in neuropsychological test performance within or outside the scanner. Noticeably estrogen did not seem to equally affect all cognitive domains, as shown by experimental studies (Gibbs, 2010).

fMRI detects changes in the blood oxygen level dependent (BOLD) signal, which is an index of the metabolic expenditures by neurons or glia during and immediately after the task. The BOLD signal is commonly accepted to reflect changes in neural activity, but interpretation should be cautious (Henderson and Greicius, 2010). Overall, it seems plausible to conclude that estrogen, alone or in combination with progestagens, positively affects brain activation during memory processing in postmenopausal women (**Tables 1**, **2**). Nevertheless the neural underpinnings of HRT effects on memory remain to be elucidated. The prefrontal cortex is of particular relevance for cognition, and the neural substrate of the estradiol-induced memory enhancement, possibly due to its sensitivity to the hormonal milieu. Future studies are needed to clarify whether HRT may lower the risk of Alzheimer's disease and protect against age-related declines in memory (Henderson and Greicius, 2010; Marjoribanks et al., 2012), as well as which duration and timing of HRT would be optimal (Daniel, 2013).

Pharmacological menopause and cognitive functioning

Gonadotropin-releasing hormone agonist (GnRHa) treatment has been used as a model for menopause to assess cognitive signatures in young women (**Table 3**). GnRH is produced by the hypothalamus and pulsatile secretion of GnRH causes the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Continuous treatment with GnRHa, on the other hand, will result in suppressed gonadal hormone production and is commonly prescribed for endometriosis, advanced breast cancer, but also to facilitate and secure subsequent safe super-ovulation during assisted reproduction therapy.

Table 3 | Studies using GnRH agonist treatment neuroimaging cognitive functioning.

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique / tracer	Test	Comparison	Results	Notes
Craig et al., 2010	30 [S:−4]	38 ± 7 40 ± 5 (26–47)	gr1: ($n = 16$) GnRH (8w) + GnRH & scopolamine gr2: ($n = 14$) placebo + placebo & scopolamine	Ph-fMRI (2x)	Visual working memory + cholinergic antagonist	GnRH vs. placebo, + anticholinergic	GnRH: ↓↓ sF (BA6+9), miF (BA9+46), iF (BA45), oF (BA10), PHG l (BA34), smG gyri r (BA40), precG (BA6), iPL (BA40), aC (BA24+31)	= Accuracy performance; ↓ response time in GnRH+scopolamine performance
Craig et al., 2009	26 [S:−4]	39 ± 2[a] 41 ± 1[a] (26–47)	gr1: ($n = 13$) baseline + GnRH (8w) + GnRH & scopolamine gr2: ($n = 13$) baseline + placebo (8w) + placebo & scopolamine	Ph-fMRI (3x)	Encoding and recognition memory + cholinergic antagonist	GnRH vs. placebo	GnRH: ↓iFG l (BA45)	= Encoding performance; ↓ recognition performance in GnRH+scopolamine
						GnRH vs. placebo, + anticholinergic	GnRH: ↓iFG l (BA45)	
Craig et al., 2008b	13 [S:−4]	39 ± 7	FP + GnRH (8w) + FP (after 6 m)	Ph-fMRI (3x)	Encoding and recognition memory	GnRH vs. FP preGnRH	[recognition:] ↓ IFG l	↓ Acccurancy of recognition performance during GnRH use
						GnRH vs. FP post GnRH	[recognition:] ↓ IFG l	
						FP preGnRH vs. FP post GnRH	[recognition:] n.s.	
Craig et al., 2008a	34 [S:−4]	39 ± 2[a] 41 ± 1[a]	gr1: ($n = 17$) FP + GnRH (8w) gr2: ($n = 17$) FP + placebo (8w)	Ph-fMRI (2x)	Visual working memory	GnRH vs. placebo	[encoding:] GnRH: ↓ sPC l/paracL?(BA5), aCC l (BA24), pC l (BA31), pCun l (BA 7), PHC l (BA35), miTG l (BA21+22+39)	= Performae
						GnRH vs. placebo across time	[encoding:] GnRH: ↓ Cb l/pCun l (BA7), pC l (BA31), FuG l (BA37)	
						GnRH vs. placebo	[recognition:] GnRH: ↓ Cb, BS	
						GnRH vs. placebo across time	[recognition:] Cb, FuG, iTG l	
Craig et al., 2007b	30 [S:−4]	38 ± 2 40 ± 1 (26–47)	gr1: ($n = 15$) FP + GnRH (8w) gr2: ($n = 15$) FP + placebo (8w)	Ph-fMRI (2x)	Verbal encoding and retrieval memory	GnRH vs. placebo	[encoding:] GnRH: ↓ mFG, iFG l (BA44, 45), aCC l, and precG r	
						GnRH vs. placebo across time	[encoding:] GnRH: ↓ mFG, miFG, iFG l, aCC l	

(Continued)

Table 3 | Continued

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique / tracer	Test	Comparison	Results	Notes
Craig et al., 2007a	10 [S:–4]	(26–47)	FP + GnRH (8w)	Ph-^{1}H MRS	NAA, Cho, Cr, ml, Glx	GnRH vs baseline	[Cho, Cho/Cr:] GnRH: ↑ dlPFC I	
Berman et al., 1997	11 [S:–4]	35 ± 7 (27–49)	GnRH (8–12w)+ GnRH+E (4–5w) + GnRH+P (4–5w)	Ph-PET [^{15}O] water	Working memory	GnRH vs. GnRH+E	E: ↑ iFG, FuG I, HG I, iTL/Cb I	= Performance
						GnRH vs. GnRH+P	P: ↑ iFG, iTG I, iPL I	
						GnRH+E vs. GnRH+P	E: ↑ HC I, mTG/iTG I	

See Appendix for acronyms/abbreviations.

GnRHa treatment results in postmenopausal levels of endogenous estradiol by the second to fourth week of administration.

Berman and colleagues performed a seminal PET study using a GnRHa-induced hypogonadism model in 1997, and found higher blood flow in the inferior frontal gyrus during estrogen- or progestagen add-back to the GnRHa treatment (Berman et al., 1997). Three subsequent fMRI studies from Craig et al., though region-of-interest based, indicated a decreased activation of the same region, i.e., the left inferior frontal gyrus, during different memory tasks, after 8 weeks of pharmacological menopause (Craig et al., 2007b, 2008a,b). Moreover, this effect was demonstrated to be reversible as reflected at re-examination six months after discontinuation of GnRHa treatment (Craig et al., 2008b). Also, restored cognitive performance has been observed after GnRHa resolution (Craig et al., 2008b), or GnRHa discontinuation plus estrogen add-back treatment (Sherwin and Tulandi, 1996).

These results essentially corroborate the neuroimaging findings on working memory (i.e., visual, encoding and recognition, verbal and retrieval memory) in postmenopausal women (**Tables 1–3**). It is likely that these effects are not a direct consequence of the GnRHa but rather an indirect effect of reduced estradiol and progesterone levels in the brain. However, though the pseudo menopause produced by GnRHa is a powerful model, the impact of short-term ovarian suppression on cognition in young premenopausal women seems to be contradictory; in some cases strong (Sherwin and Tulandi, 1996; Grigorova et al., 2006), and in other cases only marginal (Rocca et al., 2014) or absent (Owens et al., 2002).

Taken together, though limited by a region-of-interest analysis approach, pharmacological challenge that induced acute ovarian hormone suppression in premenopausal women leads to attenuation of the brain activation pattern in the left inferior frontal gyrus (**Table 3**), a region sub-serving deep processing of to-be-learned words, language and memory, typically activated during performance of executive function tasks in healthy women.

NEUROBIOLOGICAL FUNCTIONING

Neurotransmitter systems

The changes in the hormonal milieu, and particularly in estrogen levels, during the menopausal transition period are likely to modulate neurotransmitter and neuroendocrine systems, thus modifying intermediate phenotypic patterns. Such neuro-psycho-biological signatures may be involved in the mechanisms by which hormone changes may elicit cognitive dysfunction (Epperson et al., 2013), mental distress, and mental disorders (Hafner et al., 1993; Hafner, 2003; Freeman et al., 2006, 2014; Riecher-Rössler and Kulkarni, 2011). Most likely, the hormonal effects on brain function highlighted by fMRI studies are indirectly mediated through hormonal actions on neural sites serving multiple neurobiological systems (e.g., cholinergic, serotonergic, noradrenergic, and dopaminergic neurotransmitter system), as indicated by studies of primates showing estrogen-mediated neurotransmitter activities in the prefrontal cortex via alteration of cholinergic and monoaminergic innervations (Voytko et al., 2009), and emphasized by the widespread distribution of estrogen and progesterone receptors in the brain (Brinton et al., 2008; Wharton et al., 2012). The cholinergic, serotonergic,

Table 4 | Positron emission tomography, pharmaco-functional magnetic resonance imaging, single-photon emission computed tomography, and magnetic resonance spectroscopy neuroimaging studies of HRT.

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique / Tracer	Test	Comparison	Results	Notes
Kranz et al., 2014	30 [S:≥+1B/C]	55 ± 5 (47–64)	gr1 ($n = 10$): E gr2 ($n = 10$): E+P gr3 ($n = 10$): placebo	PET (2x) [carbonyl-^{11}C]WAY-100635	5-HT_{1A}	Baseline vs. E or HT	n.s.	
Epperson et al., 2012	8 [S:≥+1B/C]	53 ± 4	Tryptophan/sham depletion baseline + transdermal E_2(3–8w)	ph-fMRI (2x)	5-HT depletion + working memory	TD by ET interaction	TD [2 Back]: ↓dlPFC, miF/CG, but not after ET	= Performance
					5-HT depletion + emotion identification	TD by ET interaction	TD+ET: ↓OFC, Amy, than TD alone	= Performance
Smith et al., 2011	50 [S:+1C/2]	66 ± 4 64 ± 5 66 ± 5	HT history (within 2y of MP and ≥10y): gr1 ($n = 13$): CEE gr2 ($n = 21$): CEE+P gr3 ($n = 16$): NT	PET [^{11}C]PMP	AChE	ET vs. HT vs. NT	HT vs. NT: ↑HC l, pC	Only pC after adjustment for years of HT
Compton et al., 2008	34 [S:+1C/2]	62 ± 6 65 ± 8	gr1 ($n = 17$): E/CEE gr2 ($n = 17$): NT	SPET ^{123}I-5-I-R91150	5-HT_{2A}	NT vs. ET	NT: ↑ HC	= Performance; HC 5-HT_{2A}:-corr memory
Norbury et al., 2007	32 [S:+2]	65 ± 6 65 ± 8	gr2 ($n = 11$): MP ET (past ET +sMP) gr3 ($n = 11$): MP NT	SPET (R,R)[^{123}I]-I-QNB	m_1/m_4 mAChR	ET vs. NT	ET: ↑ striatum l, HC l, lFC, Tha	↑ Performance in executive function; Corr: E and m_1/m_4 in TC, HC l in ET
Yue et al., 2007	182 [S:+2]	66 ± 8 67 ± 8	gr1 ($n = 83$): HT (>4y, !low dose) gr2 ($n = 99$): NT	^{1}H MRS	NAA, tCR, mI	HT vs. NT	HT: ↑ NAA/tCr HC in ApoE ε4 carriers	ApoE Genotype effect
Gardiner et al., 2004	13 [S:+2(?)]		Baseline + CEE (4w) +CEE & P (2w)	SPET [99mTc]TRODAT-1	DAT	Baseline vs. ET	ET: ↑ aPu l	
						Baseline vs. HT	HT: ↑ aPu	

(Continued)

Table 4 | Continued

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique / Tracer	Test	Comparison	Results	Notes
Kugaya et al., 2003	10 [S:≥+1B]	54 ± 7	Baseline + E (10w)	PET (2x) [^{18}F]denteroaltanserin	5-HT$_{2A}$	Baseline vs. ET	ET: ↑ PFC r (BA9), iFG r (BA47), meFG r (BA6, 10), aCC r (BA32) corr +: E and 5-HT$_{2A}$in iFG r (BA44)	HT: ↑ verbal fluency and executive cognition performance, but not mood
Moses-Kolko et al., 2003	5 [S:≥+1(?)]		Baseline + E (8–14w) + E and P (2–6w)	PET (3x) [^{18}F]altanserin	5-HT$_{2A}$	Baseline vs. ET	ET: ↑ sFG r, vlPFC r, iPL l, TL l	
						Baseline vs. HT	HT: ↑ sFG, precG l, Ins l, meFG r, lOFC l, pCG r, Cun r, Tpole l, mTG, LgG l, PHG l, FuG r, meOG l	
						ET vs. HT	HT: ↑ sFG l, meOFC r, lOFC l, pCun/sPL r	
Smith et al., 2001	28 [S:+2]	64 ± 3 65 ± 4 67 ± 6	(HRT within 2y MP): gr1 ($n = 8$): E gr2 ($n = 8$): E + P gr3 ($n = 12$): NT	SPECT [^{123}I]BVM	VAChT	ET vs. NT	n.s.	= Overall performance
						ET vs. HT	ET: ↑ pC	
						yET/HT	yET/HT +corr: FC, PC, TC, aC, pC	
Robertson et al., 2001	37 [S:+2]	63 ± 10 65 ± 8	[parietal lobe]: gr1 ($n = 21$): E (CEE, $n = 5$; +P, $n = 2$) gr2 ($n = 16$): NT [Hipp]: gr1 ($n = 14$): E gr2 ($n = 12$): NT	^{1}H MRS	NAA, Cr+PCr, Cho	ET vs. NT	[Cho]: NT: ↑ PL, HC [Cho]: NT: corr −: memory in HC	No effect of ApoE genotype
Moses et al., 2000	5 [S:≥+1B]	52 ± 3	Baseline + E_2 (8–14w) + E_2 and P (2–6w)	PET (3x) [^{18}F]altanserin	5-HT$_{2A}$	Baseline vs. ET vs. HT	HT vs. baseline: ↑lOFC, pgACC, dlPFC, daCC, CER	Possible sole effect of E over time (DV_{ROI})

See Appendix for acronyms/abbreviations.

and dopaminergic systems have, in fact, been preliminarily investigated in relation to menopause, and seem to be biological mediators of positive effects of estrogens (**Table 4**) (Henderson and Greicius, 2010).

Acetylcholine (ACh). The central cholinergic system is critically involved in memory, learning and attention. Physiological aging is associated with reduction in cholinergic functional markers, such as choline acetyltransferase (ChAT), the enzyme synthetizing ACh, but with a relative preservation of cholinergic cells and terminals. Alzheimer's disease, on the other hand, is associated with drastic reductions in ChAT and diffuse degeneration of cholinergic terminals as well as loss of acetylcholine nicotinic and muscarinic receptors m1 and m4 (Gibbs, 2010; Chopra et al., 2011). Both estrogen receptors have been identified in the nuclei of the human basal forebrain, which is the source of major cholinergic innervations to the cerebral cortex, hippocampus, and hypothalamus (Gibbs, 2010). Moreover, decreased cholinergic axon density in the prefrontal cortex was found in ovariectomized monkeys compared to estrogen-only or estrogen-progesterone treated animals, as well as to control animals (Kritzer and Kohama, 1999); and cholinergic fiber density in the prefrontal, but not parietal, cortex of ovariectomized monkeys is preserved after two years of estrogen treatment (Tinkler et al., 2004). Estrogen is indeed known to provide neurotrophic support, possibly through synergistic actions with the cholinergic system, and to regulate acetylcholine release, as supported by findings in rodents and non-human primates (Voytko et al., 2009; Daniel, 2013). Thus, while low menopausal estradiol levels are likely to diminish, HRT may enhance the survival or plasticity of cholinergic cells in postmenopausal women.

A cross-sectional PET study of women who initiated HRT within 2 years of menopause, and who had used it for more than 10 years, indicated a higher cholinergic activity (8–10%), measured as acetylcholinesterase (AChE), the enzyme degrading ACh into choline and acetate, bilaterally in the posterior cingulate of combined estrogen-progestagen users compared to non-users (Smith et al., 2011). No effect on AChE was observed in the estrogen-only users compared to the non-users (Smith et al., 2011), suggesting a critical role of progesterone. On the contrary, cholinergic synaptic terminal density, assessed as binding of acethylcoline transporter (VAChT) in a SPECT cross-sectional study, was 25% higher in the parietal cortex of women treated with estrogen-only, whom initiated HRT within 2 years of menopause, compared to combined hormone users (Smith et al., 2001). Moreover, VAChT binding in frontal, temporal and parietal cortices, as well as anterior and posterior cingulate regions, was positively correlated with duration of HRT use (Smith et al., 2001), consistent with a longer exposure to gonadal steroids being associated with trophic support of cholinergic neurons. Within the cholinergic system, receptors have also been investigated using SPECT in a cross-sectional study, and higher acethylcoline muscarinic receptor m1/m4 binding was observed in postmenopausal estrogen users compared to women not receiving hormonal therapy, with greater density in the hippocampus, prefrontal cortex, striatum and thalamus (Norbury et al., 2007).

Additional proof of an interaction between estrogens and the cholinergic system has been given by two pharmaco-fMRI studies by Craig and colleagues (**Table 3**). This is in line with a protective effect of estrogen from the detrimental effects of anti-ACh challenge on attention found in humans (Dumas et al., 2006, 2008), monkeys (Voytko, 2002), and rodents (Sherwin and Henry, 2008). The former study demonstrated an additive effect of anticholinergic challenge with scopolamine on the decreased inferior frontal gyrus activation during encoding and recognition memory processing, in young women exposed to pharmacological menopause compared to placebo (Craig et al., 2009). The latter study, also using the cholinergic muscarinic receptor antagonist scopolamine, assessed visual working memory, and found more pronounced reduction of activation in frontal regions and parahippocampal gyrus in young women with pharmacologically induced menopause (Craig et al., 2010). Moreover, three-month estrogen treatment was shown to alter anticholinergic-related (i.e., both with the antimuscarinic drug scopolamine and with the antinicotinic drug mecamylamine) brain activation during a working memory task in postmenopausal women (Dumas et al., 2012b). Scopolamine challenge blocks muscarinic cholinergic receptors, and contributes to impairment of encoding of new memories; thus can be used as a pharmacological model of cognitive decline to investigate HRT-by-cholinergic interactive effects.

Altogether these studies reported an enhanced cholinergic neurotransmission in the presence of HRT, however discordantly regarding estrogen-only vs. estrogen-progestagens combined treatment (**Tables 3**, **4**). The paucity and conflicting nature of findings regarding a modulatory effect of estrogen and/or progestagens on the effects of age on neural integrity in brain regions involved in cognitive function, on age-related memory decline, and on risk for Alzheimer's disease, impede any conclusion (Marjoribanks et al., 2012; Maki, 2013). Furthermore, the effect of menopausal stage at HRT initiation on cholinergic function and related hippocampus-mediated cognitive functioning needs to be explored (Daniel, 2013). For example short-, but not long-, term hormonal treatment has been shown to enhance cholinergic function in the prefrontal cortex and hippocampus of ovariectomized rats (Gibbs, 2000).

Serotonin (5-HT). Effects of menopausal changes in hormonal milieu and HRT may be partly mediated via the serotonin pathway, as experimental animal models indicate effects of ovarian hormones on the serotonergic system (Bethea et al., 2009; Voytko et al., 2009; Sanchez et al., 2013). Three longitudinal, but small, PET studies assessing the serotonin receptor 2A (5-HT_{2A}) highlighted a general increased HRT-mediated binding potential in frontal and cingulate regions in postmenopausal women compared to baseline (**Table 4**) (Moses et al., 2000; Kugaya et al., 2003; Moses-Kolko et al., 2003). Consistently, 5-HT_{2A} receptor availability in the prefrontal cortex was higher after two months of unopposed estrogen use in two independent samples of postmenopausal women (Kugaya et al., 2003; Moses-Kolko et al., 2003). Combined estrogen-progestagen treatment for one month was associated with increased 5-HT_{2A} binding potential in the frontal, precentral, cingulate, lingual, fusiform,

parahippocampal and temporal gyrus, mainly in the left hemisphere, as well as in the insula, cuneus, and temporal pole in one study of five postmenopausal women (Moses-Kolko et al., 2003), and in the pregenual and dorso-anterior cingulate cortex, dorsolateral and lateral orbito prefrontal cortex, bilaterally, based on a region-of-interest approach, in another study of five postmenopausal women (Moses et al., 2000). Further support for a positive coupling between absolute levels of estradiol and global 5-HT_{2A} receptor availability comes from a large cross-sectional molecular imaging study in males (Frokjaer et al., 2010). Lastly, one SPECT study indicated lower hippocampal 5-HT_{2A} receptor binding to be coupled with estrogen treatment in long-term oophorectomized women, hypothesizing a down-regulation of post-synaptic receptors due to increased activity of the serotonergic pathway. However this result did not remain significant after correction for multiple comparison (Compton et al., 2008), and no firm conclusion can be derived from this finding due to the lack of correction for multiple comparisons and the low test-retest in the region of interest (Haugbol et al., 2007).

The mechanism by which HRT leads to a globally distributed $5HT_{2A}$ binding increase is not fully understood. Yet, it potentially involves hormone-mediated genomic as well as neurotrophic mechanisms. Indeed, rodent work support that 5-HT_{2A} gene transcription is affected by estradiol. In ovariectomized rats, two-week estrogen plus progesterone, however not estrogen-only, treatment was associated with increased 5-HT_{2A} mRNA in the ventral hippocampus but not in the frontal cortex (Birzniece et al., 2002). Likewise, at the protein level, 5-HT_{2A} binding in the cerebral cortex of rats was increased after two-week estrogen-only, but not combined estrogen-progesterone, treatment (Biegon et al., 1983), while in blood of depressed women was decreased after four months of hormonal treatment (Wihlback et al., 2001). Additionally, reduced 5-HT_{2A} mRNA and binding in the frontal cortex of rats was restored after a two-week estrogen treatment (Cyr et al., 1998), suggesting that hormonal withdrawal *per se* also affects 5-HT_{2A} receptor numbers. All together these findings are in line with rat studies reporting direct effects of estradiol in maintaining 5-HT_{2A} receptor levels, both after castration of male animals and after ovariectomy in female animals (Sumner and Fink, 1997, 1998; Sumner et al., 1999, 2007). Testosterone, also increase 5-HT_{2A} receptor expression, however only when converted to estradiol locally by aromatase (Sumner and Fink, 1998), again highlighting that estradiol is the main driver of $5HT_{2A}$ responses to HRT.

A recent randomized placebo controlled study of postmenopausal women found no effect of 8 weeks of HRT, of neither estrogen-only nor combined estrogen-progestagen treatment, on 5-HT_{1A} binding potential (**Table 4**) (Kranz et al., 2014). However, 5-HT_{1A} receptor binding was negatively correlated at the whole brain level with progesterone, but not estradiol, serum concentrations in postmenopausal women (Stein et al., 2014). Interestingly, an animal model of surgical menopause indicated a time-dependent effect of estrogen on this autoreceptor, with one-month, but not five-months, estrogen treatment being associated with decreased 5-HT_{1A} receptor protein expression in ovariectomized monkeys in the dorsal raphe nucleus, which is source of serotonergic neurons within CNS (Henderson and Bethea, 2008). However, combined estrogen plus progesterone administration decreased 5-HT_{1A} receptor protein expression at both time points (Henderson and Bethea, 2008). Also, 5-HT_{1A} receptor binding in the dorsal raphe nucleus has been shown to decrease after one month of treatment with either estrogen, progesterone, or a combination of both in ovariectomized monkeys (Lu and Bethea, 2002). Thus, an enhanced time-dependent effect of HRT on serotonergic neurotransmission cannot be excluded.

Additionally, using a dietary tryptophan depletion challenge, by which serotonergic tonus can be lowered transiently, serotonin-by-estrogen interaction effects were found in a small fMRI study in relation to cognitive and emotional processing in early postmenopausal women (**Table 4**). When tryptophan depleted, non-users had reduced bilateral dorsal lateral prefrontal cortex and middle frontal/cingulate gyrus activation in comparison to estrogen-only treated women; while lowered orbitofrontal cortex and amygdala activation in relation to emotion identification was observed in the estrogen users (Epperson et al., 2012). However, no interactive effect was observed between tryptophan or tyrosine/phenylalanine depletion and estrogen treatment on emotional, behavioral, and cognitive performance in healthy postmenopausal women (Newhouse et al., 2008, 2010). Women exhibited increased stress-triggered negative mood and worsened cognitive performance after a three-month estrogen administration compared to placebo, independently of monoamine depletion (Newhouse et al., 2008, 2010).

Estrogen, alone or combined with progesterone, not only modulates serotonergic function at the level of neurotransmitter synthesis, turnover, release, and receptor, but has also a neuroprotective role on serotonin neurons, as indicated by studies of monkeys (Bethea et al., 2009). The serotonin system critically modulates brain functions related to both cognition and mood, thus it is plausible to hypothesize a synergistic effect of estrogen and serotonin on cognitive functioning, emotion processing, and affective regulation (Bethea et al., 2009). Indeed, selective serotonin re-uptake inhibitors are valid alternatives to estradiol for treatment of vasomotor symptoms, i.e., hot flashes, and depressive symptoms (Al-Safi and Santoro, 2014; Joffe et al., 2014). Future studies, ideally translational, must elucidate how the serotonin system integrates sex-steroid hormone information. In particular, the relative contributions from increases and decreases in sex-steroid levels, pre- and postsynaptic receptor involvement, and the timing of the responses, are warranted to advance our understanding of how changes in sex-hormone milieu may trigger cognitive dysfunction, depressive symptoms and mental disorders.

Dopamine. Estrogens, alone and in combination with progesterone, are likely to affect the dopaminergic system too as findings in non-human primate suggest an effect, although scattered and less prominent, of estrogen on the dopaminergic system (Voytko et al., 2009). To date one sole neuroimaging study has investigated the dopaminergic system in thirteen healthy postmenopausal women, reporting that dopamine transporter (DAT) availability in women was increased in the left anterior putamen after 4 weeks of estrogen-only treatment, and in the anterior putamen after 2 weeks of combined estrogen-progestagen treatment

(Gardiner et al., 2004). Beside this study, long-term estrogen therapy has been reported to enhance dopaminergic responsivity to apomorphine in postmenopausal women (Craig et al., 2004), but further studies are needed.

Other neurochemical markers

MRS studies, through the detection of several metabolites (e.g., choline and N-acetyl aspartate), provide proxy measures of neural integrity of the brain, neuronal/glial membrane turnover and function in menopause (**Table 4**). Never-treated postmenopausal women were shown to have higher Choline (Cho)-containing compounds in the parietal lobe and hippocampus, previously identified as regions-of-interest, compared to women on HRT, and long-term visual memory was negatively correlated with Cho in the hippocampus (Robertson et al., 2001). Young women after 8 weeks of induced ovarian suppression with a GnRH agonist had higher Cho, and Cho to creatine (Cr) ratio, in the left dorsolateral prefrontal cortex (dlPFC), and borderline significant in the left hippocampus (**Table 3**) (Craig et al., 2007a). The Cho MRS peak mainly reflects the total amount of free acetylcholine, glycerophosphocholine, phosphocholine, and phosphoethanolamine; and Cho is a rate-limiting precursor in the synthesis of acetylcholine and a precursor of cell membrane phospholipids. These water soluble choline-containing compounds are proxy indicators of neuronal/glial cellular membrane turnover, and Cho levels are related to cognitive functioning and Alzheimer's disease (Chopra et al., 2011; Tayebati and Amenta, 2013). Increased Cho concentration may be due to loss of cholinergic neurons and/or increase in membrane phosphatidylcholine catabolism of neuronal cells to provide free Cho for the subsequent deficiency in acetylcholine production. In turn, membrane hypercatabolism may affect neuronal connectivity and signal transduction, causing impaired membrane processing (Chopra et al., 2011; Tayebati and Amenta, 2013).

Additionally, an increased N-acetyl aspartate (NAA) to total Cr (tCr) ratio in the hippocampus has been observed in postmenopausal women treated with low-dose HRT for more than 4 years compared to never-users, however, only among carriers of the dementia-related risk ε4 allele of the apolipoprotein E (*ApoE*) gene (**Table 4**) (Yue et al., 2007). NAA is a neuronal marker reflecting neuronal density/function and/or mitochondrial metabolism; while tCR is mainly composed of phosphocreatine (PCr) and creatine (Cr), which are associated with high-energy phosphate metabolism, and constitute a marker for phosphate metabolism. At normal conditions tCr concentration (Cr+PCr) in the brain is very stable, which is why it can be used to normalize indexes, such as NAA/tCR. A decrease of NAA indicates damage or reduction of neurons, whereas an increase of NAA implies enhancement of the neuron metabolism thus suggesting a positive effect of HRT on neuronal function and metabolism. Nonetheless, gene-by-hormonal interactions have rarely been studied in relation to mental health in menopause. In this case, genetic risk conferred by the ε4 allele of the *ApoE* gene (Yue et al., 2007), which has a dose-related effect on risk and onset age of AD, seems to interact with HRT, and call for independent replication in larger samples. Genetic markers associated with neurodegenerative disorders might have interactive or addictive effects on hormonal shifts effects.

All together these neurochemical changes provide initial evidence of a beneficial effect of HRT on neuronal markers (**Table 4**). No significant effect of HRT on the concentration of other neurometabolites (e.g., myo-inositol, glutamate and glutamine) quantified with MRS have so far been reported (Craig et al., 2007a; Yue et al., 2007). Myo-inositol (ml) is a putative glial marker which affects neuronal signal transduction and cellular osmolarity, and increased mI has been found in neurodegenerative diseases; while a decrease of the sum of glutamate plus glutamine (glx) has been related to loss of glutamatergic neurons, reduced synthesis or increased demand of glutamate as an amino acid.

BRAIN METABOLISM, RESTING STATE REGIONAL BLOOD FLOW AND FUNCTIONAL CONNECTIVITY

Moreover, positron and single-photon computed emission tomography studies of resting state brain activity indicated a stimulating, or preserving, function of hormonal treatment for the cerebral blood flow and connectivity (**Table 5**), however, regions differing in cerebral glucose metabolism overlapped poorly between studies (Ohkura et al., 1995; Eberling et al., 2000; Maki and Resnick, 2000; Rasgon et al., 2001, 2005, 2014; Slopien et al., 2003; Kenna et al., 2009). Nevertheless, it remains unclear whether greater regional cerebral blood flow (rCBF) indicates better performance. PET studies assessing the rCBF employ rCBF as a marker of local neuronal activity; however questions remain about the source and meaning of the rCBF, as other mechanisms, such as changes in cerebrovascular response (vascular tone) must also be considered. SPECT also measures rCBF, and is susceptible to hemodynamic changes, since intra-cerebral vessels are responsible for the rCBF and blood-brain barrier permeability; thus increased blood flow may be attributed to effects of estrogen on the cerebrovasculature (Ohkura et al., 1995; Eberling et al., 2000; Maki and Resnick, 2000; Rasgon et al., 2001, 2005, 2014; Slopien et al., 2003; Kenna et al., 2009). Finally, two recent studies of women at high risk for Alzheimer's disease provided evidence of differential changes in brain metabolism depending on estrogen formulation and/or estrogen-progestagen HRT (Silverman et al., 2011; Rasgon et al., 2014).

Nonetheless, HRT related brain activity and connectivity responses are not well elucidated (**Table 5**). In particular, so far no studies of resting state MRI have been published. However, lower mPFC-hippocampal connectivity at rest has been found in healthy postmenopausal women at risk for dementia who use HRT, in the presence of high plasma insulin levels (Kenna et al., 2013). Thus, studies of reproductive aging and HRT, and their relation with brain imaging of functional connectivity are needed.

EMOTION PROCESSING

The peri menopause is often accompanied by symptoms of depression and anxiety (Al-Safi and Santoro, 2014), and studies of rodents and non-human primates have attempted to investigate the neurobiology underlying postmenopausal-like affective processing (Shively and Bethea, 2004; Mueller et al., 2014). For instance, ovariectomized female rodents displayed less anxiety-like behavior when receiving estradiol replacement

Table 5 | Neuroimaging studies of brain metabolism HRT.

Study	Subjects [STRAW]	Age (y)	Hormonal treatment	Technique / Tracer	Test	Comparison	Results	Notes
Rasgon et al., 2014	45 [S:≥+1B]	58 ± 4 58 ± 6	HRT within 1y MP: gr1 ($n = 28$):HT+ (E, $n = 16$; CEE, $n = 12$) gr2 ($n = 17$): HT− (E, $n = 13$; CEE, $n = 4$)	PET (2x) FDG	rCBF	HT+ vs. HT− E vs. CEE HT+P vs. HT	HT−:↓ mPFC; iFC r, PC r HT−(E), HT+(CEE): ↓ pCU, pCC HT+(E+P): ↓ PT, pCC HT−(E+P): ↓ mFG	No effect of ApoE genotype
Kenna et al., 2009	22 [S:≥+1C/2]	55 ± 3 53 ± 4	E within 1y MP: gr1 ($n = 11$): E + P (P, $n = 9$) gr2 ($n = 11$): NT	PET [^{18}F]FDG	rCBF	HT vs. NT	HT: Thal-Ln,Thal-Cn, Ln-Cn (basal ganglia) connectivity	Used as index of DA & Choline signaling
Rasgon et al., 2005	20 [S:+2]	60 ± 7 71 ± 9	gr1 ($n = 11$): E/CEE+P gr2 ($n = 9$): NT	PET (2x) [^{18}F]FDG	rCBF	Baseline HT vs. NT +2y	n.s. NT: ↓ pCC	= Performance; no effect of ApoE genotype
Slopien et al., 2003	20 [S:≥+1B]	49 ± 5	gr1 ($n = 20$): MP baseline + E+P (1y)	SPECT HMPAO	rCBF	Baseline vs HT	HT: ↑ ventricular slices (cen prefrontal reg, low pariet reg, parieto-occip reg, upp occip reg)	
Rasgon et al., 2001	12 [S:≥+1C/2]	65 ± 9 72 ± 9	gr1 ($n = 3$): CEE+P; ($n = 1$): E gr2 ($n = 8$): NT	PET (2x) [^{18}F]FDG	rCBF	Baseline HT vs. NT +2y HT vs. NT	n.s. ET: ↑ lateral TR	No effect of ApoE genotype
Eberling et al., 2000	13 [S:+2]	71 ± 8 75 ± 6	gr1 ($n = 8$): E (≈20y) gr2 ($n = 5$): NT	PET [^{18}F]FDG	rCMRglc	ET vs. NT	?	ET: ↑ dlFC, miTG, iPL
Maki and Resnick, 2000	28 [S:+2]	66 ± 6 68 ± 6	gr1 ($n = 12$): E (+P, $n = 6$) (pastET≈15y) +2y gr2 ($n = 16$): NT (pastET, $n = 5$) +2y	PET (2x) [^{15}O] water	resting state	ET vs. NT over time	ET: ↑ mi/sTG r, iTG r, miTG l	HT: ↑ memory performance
Ohkura et al., 1995	14 [S:?]	44 ± 2 43 ± 7	past HRT for ≥1y: gr1 ($n = 9$): MP baseline + CEE (3w) gr2 ($n = 5$): MP baseline + NT (3w)	SPECT ^{123}I-IMP	rCBF	CEE and NT over time	ET: ↑ whole brain, Cb	

See Appendix for acronyms/abbreviations.

Table 6 | Functional magnetic resonance imaging studies of emotional processing during HRT.

Study	Subjects [STRAW stage]	Age (y)	Hormonal treatment	Technique	Test	Comparison	Results	Notes
RANDOMIZED CONTROLLED TRIAL								
Love et al., 2010	10 [S:≥+1B]	57 ± 1	No HT (>3m): gr1: E+P (4w) + wash-out (4w) and placebo (4w) gr2: placebo (4w) + wash-out (4w) and E+P (4w)	fMRI	Emotion processing (positive, neutral, negative stimuli)	Placebo vs. HT	[Neg] HT: ↑ OFC, OC l, precG r, pC l Placebo: ↑dlPFC l, postcG r, daC [Pos] Placebo: ↑ mFC l	= Behavior
CROSS-SECTIONAL STUDIES								
Shafir et al., 2012	52 [S:≥+2]	67 ± 6 65 ± 5 65 ± 5	HT (within 2y of MP and ≥10y): gr1 ($n = 15$): CEE gr2 ($n = 20$): CEE+P gr3 ($n = 17$): NT	fMRI	Emotion processing (positive, neutral, negative stimuli)	NT vs. HT	n.s.	↑ Time for picture rating in HT; ↑ Accuracy in current HT
						NT vs. ET	NT: Pos–Neu: ↑ mFG l (BA10), aC (BA24+32) ET: Neg–Neu: ↑entorhinal cortex r	
						NT vs. HT	NT: Pos–Neu: ↑Ins r	
						Current vs. past HT	Current HT: Pos–Neu: ↑HC r	
Frey et al., 2010	11 [S:≥−2]	51 ± 6 (40–60)	/	fMRI	Emotion regulation	High- vs. low-emotional conflict resolution	High-conflict resolution: dlPFC (BA42+40+13+9+7+Pu+30+6+Cau+30+39)	! No comparison group

See Appendix for acronyms/abbreviations.

(Mueller et al., 2014). In humans, increased stress-triggered negative mood after a three-month estrogen administration was noted in healthy postmenopausal women (Newhouse et al., 2008), and in older women (Dumas et al., 2012a). However, HRT has been associated with decreased depressive symptoms in menopausal women (Zweifel and O'Brien, 1997), though depending on psychiatry history, age and study design; while other studies, such as the Women's Health Initiative study, provided conflicting evidence (Henderson and Greicius, 2010). Moreover, free estradiol levels were positively correlated with mood in a recent large study of healthy women during early postmenopause (Henderson et al., 2013), but again HRT, in particular current use, was associated with symptoms of depression and anxiety in two large cohorts of peri-menopausal women (Toffol et al., 2013), however estradiol is generally recognized to have positive effects on mood (Soares, 2013).

Neuroimaging studies of emotion processing in menopausal women are essentially lacking, and findings are thus far inconclusive (**Table 6**). A randomized placebo-controlled cross-over study including ten postmenopausal women portrayed an increased activation, though in different regions, during negative emotional stimuli processing, in both the placebo and estrogen-progestagen treated groups, and higher activation of the left medial frontal cortex during positive stimuli processing in the placebo group (Love et al., 2010). Later, a larger cross-sectional study of long-term HRT users also showed puzzling activation in estrogen treated women and non-users, and in current vs. past HRT users (Shafir et al., 2012). While Love et al. found no effect on performance of the task (Love et al., 2010), Shafir et al. found a slower response time in HRT users, but higher accuracy in picture rating among current users compared to past users (Shafir et al., 2012). In addition, greater regional activity in the dorsal lateral prefrontal cortex during high conflict resolution was found in one study investigating emotion regulation in eleven peri- and postmenopausal women (Frey et al., 2010).

Monoaminergic projections throughout the prefrontal and parietal cortices are likely mediating the effect of estrogen and progestagens on emotion and mood. Cortical and limbic structures are key regulators in emotion processing; principally the dorsolateral prefrontal cortex, playing a role in the suppression of emotion, was less activated during negative emotional stimuli in estrogen-progestagen treatment compared to placebo (Love et al., 2010), and the amygdala, a key region in emotional learning, had diminished emotion-induced activation after estrogen-only treatment in the presence of dampened serotonergic function (Epperson et al., 2012).

Interestingly, in relation to the "critical window" hypothesis, are the results of a functional magnetic resonance imaging study of older women, above the age of 65 years, comparing current long-term HRT users vs. past short-term users, and fundamentally indicating no difference in emotion-induced brain activity (Pruis et al., 2009). To sum up, the precise alterations in neural activity underlying the effects of HRT on mood in menopausal women remain to be understood, since a quarter of heterogeneous neuroimaging studies is not sufficient neither to tackle the question nor to draw conclusions.

TESTOSTERONE TREATMENT

Testosterone treatment in postmenopausal women has been investigated by only one study. An fMRI study of nine postmenopausal women indicated a decreased activation of frontal and lingual gyri during verbal fluency, and of the parietal lobe and precuneus during mental rotation, after 6 months of transdermal testosterone treatment (Davis et al., 2013). Higher endogenous testosterone has been associated with superior performance in verbal fluency, and spatial and mathematical abilities; thus these preliminary results might indicate a more efficient brain functioning in response to cognitive tasks.

Testosterone levels are about 65% lower during menopause, and though ovarian production of testosterone continues after natural menopause, very low levels are gradually reached. Testosterone treatment is generally used for menopausal women with female sexual dysfunction, i.e., diminished sexual interest. Thus, knowledge of effect of testosterone replacement therapy in the brain should also be achieved, although it also remains to be established if putative effects are directly attributable to testosterone or rather due its conversion to estradiol, as has been showed convincingly for e.g., the 5-HT_{2A} receptor responses to HRT (Sumner and Fink, 1998).

DISCUSSION

Menopause is the most influential biological and health-related event for most middle-aged women. During the perimenopause (Harlow et al., 2012), which can last several years, the level of estrogen fluctuates substantially but gradually declines. The lowered estrogen levels are often accompanied by sometimes debilitating vasomotor symptoms (Harlow et al., 2012; Al-Safi and Santoro, 2014). The decision to use HRT or not represents a major concern for afflicted women and their clinicians. Effects of the menstrual cycle and pharmacological contraceptive treatment on the central nervous system, inferred from behavioral and neurophysiological activity changes in response to cognitive and emotional stimuli, indicate a role of estrogen and progesterone on brain function in healthy fertile women (Mueller et al., 2014; Toffoletto et al., 2014). Alike, menopausal hormone changes are likely to modulate neuronal activity and contribute to age-related memory loss as well as the development of disorders such as dementia or major depression (**Tables 1–6**).

It is tempting to conclude that the present findings reflect true alterations in neural activity in the face of a hormonal perturbation (**Tables 1–6**). However, the interpretation of the results presented in this review remains a matter of debate, since less activation during task performance can be interpreted either as a more efficient use of neuronal resources or as an impaired state in which adequate activation of resources cannot be executed. Vice versa, higher activation can be interpreted as beneficial, or imply a compensatory response, or a less efficient use of resources (Henderson and Greicius, 2010). Interestingly, most of the effects were observed after a relatively brief time of HRT use. Thus, the optimal duration, or dose of HRT, or timing of treatment start, necessary to maintain a premenopausal cerebral state or to trigger neuroplasticity in mature women, is unknown.

Peri- and postmenopause are marked not only by vasomotor symptoms, but also by cognitive and emotional complaints that

affect quality of life and overall functioning; however the degree to which the decline in estrogen and/or progesterone production is responsible is not yet clear. Increasing evidence from observational and HRT studies of postmenopausal women supports the influence of ovarian hormones on cognition and mood both at the behavioral and neural level, although it remains ambiguous whether this influence is positive or harmful (Henderson and Greicius, 2010). In the reviewed studies, estrogen altered the activation of brain structures underlying memory performance, particularly the fronto-cingulate region; however there appears to be a puzzling dissociation between neuropsychological performance and neural activity, since many studies that found a hormonal-dependent effect in neuroimaging data did not observe neuropsychological alterations across hormonal conditions (**Tables 1–3**). Also, cerebral activity changes are task-dependent, as shown by experimental models (Sherwin and Henry, 2008; Gibbs, 2010), and may as well be biased by differences in education and health of the women. Longitudinal observations are hence indispensable to identify if, and at which critical age, the metabolic decline may become appreciable (Henderson and Greicius, 2010). Thus, abnormal activation when associated with normal task performance may reflect compensatory cerebral metabolic activity in response to a subtle/subclinical loss of cognitive capacity, and precede cognitive impairment in individuals at increased risk for neurodegenerative and psychiatric disorders. However, an alternative explanation to group differences in activation patterns during memory processes may be that postmenopausal women activate a distinct cognitive network. Overall, it seems plausible to conclude that estrogen, alone or in combination with progestagens, positively affects brain activation during memory processing in postmenopausal women (**Tables 1**, **2**), likely via interactions with basal forebrain cholinergic projections (Gibbs, 2010). This suggestion is also in line with findings of preserved visual memory in ovariectomized monkeys treated with estrogen, alone or in combination with progesterone, compared to placebo-treated ovariectomized monkeys (Voytko et al., 2009). In contrast, results regarding cerebral functional responsiveness to emotional stimulation are lacking and far from being consistent (**Table 6**).

Furthermore, studies of neurochemical pathways have pinpointed the cholinergic, serotonergic, and dopaminergic systems as biological mediators of estrogen influences on the brain (**Table 4**). Cholinergic and monoaminergic wide-spread projections to prefrontal cortical regions interactively mediate several functions ranging from working memory to emotion processing. For instance, quantitative and qualitative differences in density of axons immunoreactive to ChAT, dopamine ß-hydroxylase, tyrosine hydroxylase and serotonin in the dorsal medial prefrontal cortex was found in ovariectomized monkeys compared to control animals, and following 1 month treatment with estrogen-only or estrogen-progesterone (Kritzer and Kohama, 1998, 1999). The interplay between these neurotransmitter systems is of relevance for schizophrenia and depression, two mental disorders characterized by sex differences in their occurrence, and their interaction with hormonal changes is likely to impact treatment response.

More, knowing that cerebral blood flow decreases with age, positron, and single-photon computed emission tomography studies suggested a preservative/stimulating function of estrogen for the cerebral blood flow (**Table 5**). In view of these findings (**Tables 1–5**), and of studies of monkeys (Voytko et al., 2009), a putative neuroprotective role of estrogen in neuronal integrity maintenance and cognitive function preservation against regional cerebral metabolic decline could thus be anticipated/predicted, also considering meta-analytical findings reporting an effect of about one-third reduction in the risk of Alzheimer's disease among HRT users (Yaffe et al., 1998; Hogervorst et al., 2000; LeBlanc et al., 2001; Nelson et al., 2002). However, as highlighted by the Women's Health Initiative project, timing is likely to be critical for positive HRT effects on cognition (Henderson and Greicius, 2010).

Of special interest in relation to neurogenesis and synaptic plasticity is the hippocampus, a crucial region in memory and learning (Gibbs, 2010). Preclinical research has highlighted several effects of estrogen on structure and function of the hippocampus in animal experiments, also via effects on cholinergic projections from the forebrain, ranging from neurotrophic to neuroplastic to neurobehavioral effects (Voytko et al., 2009; Wnuk et al., 2012; Daniel, 2013). The hippocampus is atrophied and functionally impaired in Alzheimer's disease and in the presence of a history of repeated depressive episodes, neurobiological features which are linked to cognitive function. Larger hippocampal volume in HRT users has been indicated by magnetic resonance imaging studies, with the first evidence reported by Eberling et al. (2003). Several experimental and clinical studies have in fact provided evidence of a positive modulatory effect of estrogen use on hippocampal anatomy, though aversive or absent effects have also been reported (Erickson et al., 2005, 2010; Boccardi et al., 2006; Lord et al., 2008; Espeland et al., 2009; Wnuk et al., 2012). Discordance of the findings and lack of longitudinal randomized controlled trials leaves room for equivocal conclusions on the effects of HRT on hippocampus morphology. However, several neuroimaging studies indicated an enhancing effect of HRT on hippocampus function (Maki and Resnick, 2000; Robertson et al., 2001; Gleason et al., 2006; Norbury et al., 2007; Berent-Spillson et al., 2010; Maki et al., 2011; Smith et al., 2011; Shafir et al., 2012), suggesting potential positive estrogen-mediated effects on neuronal survival and plasticity through the cholinergic system (Daniel, 2013). Thus hippocampal, together with prefrontal cortical, function is a strong candidate substrate mediating the hormonal effects on cognition.

Estrogens interact with neuronal networks at many levels and affect brain development and aging (Sherwin and Henry, 2008). The modulation by estrogen may therefore account for hormonal regulation of cognition and mood, as for instance women have increased risk for developing Alzheimer's dementia and major depression compared to men, but the pathophysiological mechanisms behind these differences are unknown (Henderson and Greicius, 2010). Non-invasive measurement of brain function through neuroimaging techniques is advantageous compared to traditional behavioral measures. The BOLD signal and the rCBF are proxy outcomes, endpoints which can be objectively measured and are potentially related to a clinical phenotype, such as working memory (Henderson and Greicius, 2010). Biological relevance could thus be inferred by the effect of HRT on neuronal activity; consequently the question whether certain intermediate

phenotypes can be classified as endophenotypes (Lenzenweger, 2013) for neurodegenerative and psychiatric disorders occurring during menopause remain to be addressed. Neuroimaging genetics of the peri- and postmenopause and HRT use is required to provide knowledge about constitutional vulnerability to hormonal changes in relation to mental health (e.g., the first neuroimaging genetic study of premenstrual dysphoric disorder, Comasco et al., 2014), as well as molecular research will be essential to elucidate epi-genotype-mediated mechanisms. To date there is a lack of focus on genetic correlates of menopausal hormonal effects, with only four studies having considered the *ApoE* genotype (Rasgon et al., 2001, 2005; Robertson et al., 2001; Yue et al., 2007). For instance polymorphisms in the estrogen receptor alpha and beta genes (*ESR1* and *ESR2*) have been associated with cognitive impairment in old women in two large longitudinal studies (Yaffe et al., 2002, 2009), but their potential intermediate neuronal correlates have not been investigated. Additionally, the modulatory association between the catecholamine degrading enzyme catechol-O-methyltransferase (*COMT*) and dorso and ventro lateral prefrontal cortex and supragenual anterior cingulate cortex activation during working memory has been demonstrated in schizophrenia (Rasetti and Weinberger, 2011), but remains to be explored in relation to menopausal cognitive decline.

Future studies will thus need to investigate whether putative intermediate phenotypes are involved in the causal pathway between genes and psychopathology, besides having a mere associative or consequential relationship with the illness, as well as the heritability of the endophenotype(s) (Lenzenweger, 2013). By means of such approach, differences between statistical vs. biological significance can be further discerned.

Widespread classical nuclear (i.e., estrogen receptor α and β, progesterone receptor A and B), and non-classical membrane-associated (e.g., G protein-coupled estrogen receptors and the progesterone receptor membrane component 1), receptors are targeted by estradiol and progesterone, thus mediating their effects on brain structure as well as cognitive-affective processing (Brinton et al., 2008; Wharton et al., 2012). Remarkably, estrogen replacement treatment has been the most studied (**Tables 1–6**), even though it is far less often used compared to combined estrogen-progestagen treatment. Estrogen-only treatment is prescribed only for hysterectomized women, as the progestagen addition is needed for protection against endometrial hyperplasia and cancer. Moreover, two recent studies of women at high risk for Alzheimer's disease provided evidence of differential changes in brain metabolism depending on estrogen formulation and/or estrogen-progestagen HRT (Silverman et al., 2011; Rasgon et al., 2014). Lately, a study found a positive correlation between serum progesterone, but not estrogen, levels and verbal memory and global cognition in healthy women during the early postmenopause (Henderson et al., 2013). Thus, potential cumulative neuroprotective or counteracting effects of progestagens—via its metabolites or indirectly via type-A γ-aminobutyric acid receptor signaling modulation—still need to be elucidated.

Importantly sampling differences between the studies also need to be considered since a gynecological clinic-based sample (e.g., women who underwent oophorectomy with or without hysterectomy, i.e., surgical menopause) is likely to differ from a sample of asymptomatic healthy menopausal women (Rocca et al., 2014). Furthermore, natural menopause is typified by a gradual decline of estradiol and androgen levels until circulating levels are exclusively maintained by peripheral conversion of adrenal steroids (Harlow et al., 2012; Al-Safi and Santoro, 2014). On the contrary, surgical menopause causes a drastic and complete cessation of both estradiol and testosterone secretion. Finally, efforts to stage reproductive aging according to standardized nomenclature and classifications, as the Stages of Reproductive Aging Workshop (STRAW) criteria, should be made to facilitate comparisons between studies (Harlow et al., 2012).

Hormonal supplementation in peri- and postmenopausal women remains a source of great health concern. The so-called "window of opportunity" or "critical period" hypothesis cannot be rejected based on the present results, but it is expectable that timing of HRT (i.e., age and proximity to menopause when HRT is initiated) will imply a different brain response, and that chronological and reproductive aging matter too (Henderson and Greicius, 2010; Daniel, 2013; Maki, 2013; Rocca et al., 2014). A critical threshold below which the function of neurobiological systems remains impaired by loss of ovarian function and cannot be triggered by exogenous hormonal supplementation is indeed plausible.

CONCLUSIONS

Questions to-be-answered:

I. What are the neurobiological mechanisms underlying menopause hormonal transition and HRT effects in the brain?
II. Are there synergistic or counteracting effects of estrogen together with progestagens and/or androgens?
III. Which dosage and combination of HRT should be prescribed?
IV. If and when should HRT be initialized and terminated to be most efficient in optimizing women's mental health?

Collectively, neuroimaging studies indicate altered brain activation patterns by estrogen-only or estrogen-progestagen treatment in therapeutic doses in postmenopausal women, corroborating preclinical findings of effects of estrogen on neural structure and function in mature animals. These results call for further well-powered randomized-controlled multi-modal neuroimaging studies, using a battery of standardized neuropsychological tests, as well as for complementary investigation on the correlated molecular mechanisms of reproductive aging and HRT in preclinical models.

KEY CONCEPTS

- Menopausal transition: period in a women's life lasting several years around the age of fifty where ovarian hormone levels, estrogens and androgens, fluctuate and decrease until menopause occurs with the termination of the menstrual cycle, and post-menopause is established; this physiological transitional hormonal change (peri-menopause), as well as surgically

induced menopausal transition, are of relevance to the aging female brain.

- Hormonal replacement therapy (HRT): exogenous supplementation of estrogen and/or progesterone given during perimenopause to treat vasomotor symptoms. The potential protective and/or aversive effects, and the biological mechanism of action of HRT, as well as the effects of interrupting HRT, on cognitive and emotional functioning remain largely elusive.
- Window of opportunity: crucial period of time spanning from peri- to post-menopause during which HRT could be used with beneficial effects on women's physical and mental health.
- GnRHa hormone manipulation: treatment with a gonadotropin releasing hormone agonist that initiates a biphasic response, which initially stimulates ovarian sex-steroid hormone production and subsequently induces a pharmacological pseudo-menopausal state, which is established by the second to fourth week of administration, with a complete cessation of both estradiol and testosterone secretion. It provides a model of early menopause and menopausal transition.

AUTHOR CONTRIBUTIONS

Erika Comasco, Inger Sundström-Poromaa, and Vibe G. Frokjaer: study conception; literature acquisition; data interpretation; drafting the work; revising the work critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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APPENDIX

ACRONYMS/ABBREVIATIONS IN THE TABLES

aC: anterior cingulate; aCC: anterior cingulate cortex; AChE: acetylcholinesterase; BS: brain stem; Cau: caudate; Cb: cerebellum; CEE: conjugated equine estrogen; CER: cerebellar cortex; CG: cingulate gyrus; Cn: caudate nucleus; Cun: cuneus; daCC: dorsal anterior cingulate cortex; dlFC: dorsolateral frontal cortex; dFG: dorsal frontal gyrus; dlPFC: dorsolateral prefrontal cortex; EC: entorhinal cortex; E: estrogen; FC: frontal cortex; FDG: fluorodeoxyglucose; FG: frontal gyrus; FL: frontal lobe; FP: follicular phase; FuG: fusiform gyrus; gr: group; HC: hippocampus; HERA: hemisphere encoding retrieval assimetry; HG: hippocampal gyrus; HMPAO: hexamethylpropylene-amino oxime; HT: hormonal treatment (E+P); Hy: hypothalamus; iF: inferior frontal; iFC: inferior frontal cortex; iFG: inferior frontal gyrus; Ins: insula; ipFG; inferior posterior frontal gyrus; iPL: inferior parietal lobe; iTG: inferior temporal gyrus; iTL: inferior temporal lobe; LgG: lingual gyrus; Ln: lentiform nucleus; lOFC: lateral orbito frontal cortex; mAChR: muscarinic acetylcholine receptors; MB: mammillary body; MF: medial frontal; mesial-TR: mesial temporal region; mFC: medical frontal cortex; meFG: medial frontal gyrus; meOFC: medial orbitofrontal cortex; meOG: medial occipital gyrus; miF: middle frontal; miFG: middle frontal gyrus; mFL: medial frontal lobe; miTG: middle temporal gyrus; MP: menopause; n: number of individuals; Neg: negative pictures; Neu: neutral pictures; NT: no treatment; OC: occipital cortex; oF: orbital frontal; OFC: orbitofrontal cortex; OL: occipital lobe; P: progesterone/progestins; pC: posterior cingulate; PC: parietal cortex; pCC: posterior cingulate cortex; PCL: paracentral lobule; pgG: postcentral gyrus; pCun: precuneus; paracL: paracentral lobule; PHG: parahippocampal gyrus; PL: parietal lobe; pgACC: pregenual anterior cingulate cortex; pIns: posterior insula; pCG: posterior cingulate gyrus; PFC: prefrontal cortex; PL: parietal lobe; Pos: positive pictures; post-cG: postcentral gyrus; pPHG: posterior para-hippocampal gyrus; pre-cG: precentral gyrus; Pu: putamen; rCMRglc: regional cerebral glucose metabolism; sF: superior frontal; sFG: superior frontal gyrus; smG: supra-marginal gyri; sPC: superior parietal cortex; sTG: superior temporal gyrus; STRAW: Stages of Reproductive Aging Workshop [ranging from −5 to +2] (Harlow et al., 2012); TC: temporal cortex; TD: tryptophan depletion; thal: thalamus; TL: temporal lobe; TP: temporal pole; TR: temporal region; VAChT: vesicular acetylcholine transporter; vlPFC: ventrolateral prefrontal cortex; vpTL: ventral posterior temporal lobe.

[¤]SEM: standard error of the mean.

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On the effects of testosterone on brain behavioral functions

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Testosterone influences the brain via organizational and activational effects. Numerous relevant studies on rodents and a few on humans focusing on specific behavioral and cognitive parameters have been published. The results are, unfortunately, controversial and puzzling. Dosing, timing, even the application route seem to considerably affect the outcomes. In addition, the methods used for the assessment of psychometric parameters are a bit less than ideal regarding their validity and reproducibility. Metabolism of testosterone contributes to the complexity of its actions. Reduction to dihydrotestosterone by 5-alpha reductase increases the androgen activity; conversion to estradiol by aromatase converts the androgen to estrogen activity. Recently, the non-genomic effects of testosterone on behavior bypassing the nuclear receptors have attracted the interest of researchers. This review tries to summarize the current understanding of the complexity of the effects of testosterone on brain with special focus on their role in the known sex differences.

Keywords: androgen, cognition, emotions, hippocampus, behavioral neuroendocrinology

INTRODUCTION

Despite current efforts of the European commission to combat gender issues with respect to gender equality, men and women are different in several important aspects (Cahill, 2014). These aspects include cognitive functioning and behavioral traits. Some of these may be socially induced, but scientists have showed on intact animals that other factors such as genetics and gender itself are mostly responsible forthe sex differences in behavior and cognition. Therefore, the current research strategies are calling for including both males and females in the research in order to report the possible gender differences (Ruigrok et al., 2014). Indeed, the exact mechanisms and reasons of sex differences in brain structures that mediate some of these functional dissimilarities are unknown. Genetics and endocrine factors are the most prominent biological explanations and are interconnected. Testosterone is the major male sex hormone. It is present in women, although in much lower concentrations. Testosterone has also been intensively studied in relation to sex differences and behavioral functions. This review focuses on physiology of testosterone to give the reader understanding of the mechanisms and complexity of testosterone action and then tries to summarize the studies and experiments focusing on the functional changes in anxiety, depression, spatial abilities and memory. Readers interested in sex differences and brain structures might find the needed information in the recently published focused review (Filova et al., 2013).

TESTOSTERONE PHYSIOLOGY

Testosterone is produced mainly in Leydig cells of testes in males, and in ovaries in females. In both, testosterone can be synthetized in the adrenal gland cortex (Burger, 2002; Dohle et al., 2003). However, in addition to the classic steroidogenic organs such as gonads, adrenals and even placenta, the active biosynthesis of steroids also occurs in the brain (Mellon et al., 2001). This synthesis can be either *de novo* from the cholesterol, or testosterone is derived from classical steroids as is deoxycorticosterone or progesterone, which enter through blood stream into nervous system. The latter one depends on the enzymatic ability of the neural region or cell. The key regulatory enzyme is Steroidogenic acute regulatory protein (StAR) (Miller and Auchus, 2011). This phosphoprotein mediates the transfer of cholesterol from the outer to the inner mitochondrial membrane, from where cholesterol can be further processed by corresponding enzymes. The StAR gene is expressed solely in the steroidogenic tissues. However, StAR mRNA expression in a rat brain was first shown by Furukawa (Furukawa et al., 1998) and confirmed in humans and mouse brains in several regions by immunohistochemistry.

The complexity of testosterone mechanism of action is underlined by its metabolism and steroid nature. The classical view suggests genomic mechanism, i.e., after translocation into cytoplasm, testosterone binds the androgen receptor, and subsequently after transportation into the nucleus it binds on the hormone response element at DNA, where it activates or silences the expression of genes and subsequent protein synthesis (Tsai and O'malley, 1994). During recent years, a new pathway for non-genomic mechanism was shown. This can include activating the membrane receptors and thus activating the second messengers, or after translocation to the cell, testosterone can either directly activate second messenger intracellular cascade, or can bind to its respective receptor and as a complex of hormone-receptor it

can activate the second messenger cascade (Michels and Hoppe, 2008). Additionally, testosterone can be changed into either estradiol by aromatase or into dihydrotestosterone by reductase. The pathway depends on the enzymatic equipment of the cells.

The synthesis of sex hormones is ultimately controlled by gonadotropin-releasing hormone (GnRH), which is produced by the hypothalamus and which stimulates the pituitary gland to release luteinizing hormone and follicle stimulating hormone, where LH increases the expression of StaR protein in target cells (Ubuka et al., 2014). GnRH secretion in adulthood is pulsatile and highest during sleep with subsequent highest peaks of testosterone to be during the early morning hours (Lord et al., 2014). Nevertheless, the testosterone levels decline gradually with aging, mainly due to the attrition of Leydig cells and hypothalamic GnRH pulse generation slow down. Rapid drop can be observed in the 6th decade of life in males.(Basaria, 2013). A higher incidence of mood disorders that occurs with aging is then related to decreased testosterone and/or other androgens. However, not all studies agree with this simple explanation. Sartorius et al. showed, that there was no decline in testosterone levels in males who self-reported to be in very good health. Indeed, a subgroup of patients who were smoking and/or obese was associated with age related decline in serum androgens (Sartorius et al., 2012). Similarly, Camacho et al. reported that lifestyle factors and body weight were more important in maintaining the plasma testosterone levels than aging itself (Camacho et al., 2013).

In any respect, the causal role of testosterone deficiency and behavioral disorders including effect on cognitive abilities is still debated. Therefore, in the next chapters we will try to summarize the main experimental studies on individual behavioral traits.

ANXIETY

Our daily decision-making as well as response to stress is our everyday experience. Indeed, many factors contribute and even more factors modify the decision-making and stress reaction, with anxiety level to be one of them. Nevertheless, it has clearly been shown, and recently reviewed that women show higher anxiety in comparison to men (Mchenry et al., 2014). From all behavioral parameters, the anxiety seems to be most sensitive to testosterone. The most cited paper analyzing the effects of testosterone on anxiety in mice has shown in several experiments that testosterone—either endogenous or exogenous decreased anxiety in elevated plus maze (Aikey et al., 2002). In addition, the same study showed that this anxiolytic effect of testosterone is dose-dependent and very likely mediated by 5-alpha reductase that reduces testosterone to dihydrotestosterone. The study was conducted in male mice, but similar anxiolytic effects of single testosterone administration resulted in reduced fear of healthy women (Van Honk et al., 2005). In rats, a single testosterone injection did not reduce anxiety, however, a repeated administration had anxiolytic effects tested by the burying behavior test (Fernandez-Guasti and Martinez-Mota, 2005). A possible mechanism can include the androgen receptor, as its blockade has been shown to prevent the testosterone-induced anxiolysis. Similar results were obtained in our experiment. However, the anxiolytic effect was observed only in the light-dark box. We were not able to reproduce the anxiolytic effects of testosterone in the elevated plus maze and in the open field (Hodosy et al., 2012). On the other hand, flutamide alone had anxiolytic effects in the open field. This suggests that the association between testosterone and anxiety might not be linear. A number of experiments on gonadectomized rats from the lab of professor Frye further showed that dihydrotestosterone 3-alpha metabolites can be the mediators of testosterone anxiolytic effects (Edinger and Frye, 2004, 2005). In addition, blockade of the dihydrotestosterone transformation to 3-alpha androstanediol by a 3-alpha hydroxysteroid dehydrogenase inhibitor prevented the anxiolysis (Frye and Edinger, 2004). Age-related decline in cognitive and affective functions was associated with lower concentrations of testosterone metabolites in the hippocampus. Again, this effect blocked by administration of 3-alpha metabolites administration (Frye et al., 2010). Another mechanism of anxiolytic effect of testosterone was explained in recently published experiment, where exogenous or endogenous opioids could modulate anxiolysis (Khakpai, 2014). In this study, the gama aminobutyric acid systemthat has been proposed in the past, on the other hand, did not alleviate the anxiety level (Roohbakhsh et al., 2011). An important determinant of the postnatal association between anxiety and testosterone or its metabolites might be prenatal stress. Stress induced during gestation resulted in both, reduced testosterone and increased anxiety of the adult offspring (Walf and Frye, 2012). Taken together, the results are consistent and despite differences in the methodology it seems clear that testosterone reduces anxiety in both genders. Its higher concentrations in men might be the reason for the sex differences in anxiety. However, a very important study in rhesus monkeys showed that pharmacological castration reduced and testosterone supplementation normalized anxiety levels (Suarez-Jimenez et al., 2013). A result that is in contrast to the majority of literature of experiments in rodents. Of course, this discrepancy might be discussed with major differences in the methodology—other behavioral tests, and in the intervention—surgical vs. pharmacologic castration. But in general, experiments on monkeys are more relevant to human behavior and, thus, this study must not be overseen. Some of the animal experiments on testosterone and anxiety are summarized in **Table 1**.

DEPRESSION

Although the depressive disorder is more prevalent in females (Bebbington, 1996) when compared to males, the prevalence of depression in males increases with age (Khera, 2013) as plasma testosterone drops. Consequently, many experiments and studies were performed to confirm the causative role of testosterone decline in depression pathogenesis. Indeed, these studies were not triggered by the lone fact of testosterone decline and sex difference in prevalence of depressive disorders. In depressive disorders with decreased libido and low testosterone, the androgen hormone replacement therapy was at least as effective as serotonin reuptake transporters (Kranz et al., 2014). Further it was investigated that testosterone can modulate serotonergic transmission, where serotonin plays a crucial role in depression development (Jovanovic et al., 2014). But it is not only the prevalence of depression that differ between sexes. In a study on opposite sex twins, it has been demonstrated that also the etiology of depression

Table 1 | Selected animal studies analyzing the relationship between testosterone and anxiety.

Species	Strain	Gender	Timing	Intervention	Maze	Mechanism	Outcome	References
Rattus norwegicus	Wistar	Males	Young adults	Intact rats administered single injection of testosterone propionate or flutamide	Open field L/D box EPM	Flutamide as androgen receptor blocker administered	Testosterone ↑ time in L/D box by 80% vs. Control group, probably through androgen receptor; (anxiolytic effect)	Hodosy et al., 2012
Mus musculus	129:C57BL/6J	Females	Perinatal period	OVX at 28th day postnatally and capsule insertion with TP; tested on PD 67-78	Tail ST Marble burying L/D box	Organizational or activational effect of TP administration	Testosterone groups showed ↑ immobility after TP administered early postanatally; ↓ marble burying in all TP groups; no differences in L/D box; (depressive and anxiogennic effect)	Goel and Bale, 2008
Mus musculus	C57B1/6J-NHsd	Males	Young adults	Daily injections of testosterone propionate ip	Condition place preference (L/D box)	Observational/ pharmacological	Testosterone did not increase L/D transitions (no effect); nandrolone showed decreased transitions by 30% at low doses; (anxiogenic effect)	Parrilla-Carrero et al., 2009
Rattus norwegicus	Long Evans	Males	Perinatal	Tfm and WT rats gonadectomized at postnatal day 1 and at postnatal day 120; further either GDX or sham with testosterone capsule implantation	Open field NOR L/D box EPM	Organizational or activational effect	Neonatal castration ↓ anxiety traits in all tests; no difference between Tfm and WT; (anxiolytic effect that is AR independent)	Zuloaga et al., 2011
Macaca mulatta	Rhesus monkey	Males	Young adults	GnRH-agonist injection to suppress testosterone secretion; then either oil or testosterone im	Modified human intruder test	Observational/ pharmacological	Testosterone increased anxious behavior when compared GnRH agonist by 300%, but not vs baseline; (anxiogenic effect)	Suarez-Jimenez et al., 2013
Rattus norwegicus	Long-Evans	Males	Young adults	Gonadectomized and capsules with DHT or sham implantation	Open field EPM Defensive freezing	Observational/ pharmacological; indomethacin blockade of DHT conversion to 3-alpha-diol in hippocampus	Intact and DHT replaced more active, exploratory and more time freezing than GDX; (anxiogenic effect of DHT)	Frye and Edinger, 2004

(Continued)

Table 1 | Continued

Species	Strain	Gender	Timing	Intervention	Maze	Mechanism	Outcome	References
Rattus norwegicus	Long-Evans	Males	Young adults	SHAM, GDX and GDX+testosterone supplementation	Open field Social interaction Defensive burying Paw lick Emergence test EPM	Observational/ pharmacological study	Testosterone ↑ time in the open arms of EPM vs GDX but not intact; (anxiolytic effect)	Frye and Seliga, 2001
Rattus norwegicus	Long-Evans	Males	Young adults	GDX + testosterone, DHT and 3-alpha diol capsules	EPM Open field Defensive burying Inhibitory avoidance	Observational/ pharmacological study	Systemic and intrahippocampal testosterone decreased anxiety in open field by 250% and EPM by 200% and decreased fear behavior by 28%; The effect of testosterone was not higher than in DHT or 3-alpha diol administration	Edinger and Frye, 2004
Rattus norwegicus	Wistar	Females	Young adults	Testosterone Picrotoxin Formestane Tamoxifen	Defensive burying Open field	Pharmacological intervention, and androgen/estrogen receptor blockade	Testosterone reduced cumulative burying time vs oil by 35%; no difference in open field and burying latency (anxiolytic effect of testosterone); effect mediated through androgen metabolites, not aromatization to estradiol	Gutierrez-Garcia et al., 2009

Tfm, testicular feminization mutation; WT, wild type; AR, androgen receptor; PD, postnatal day; OVX, ovariectomy; GDX, gonadectomy; TP, testosterone propionate; DHT, dihydrotestosterone; EPM, elevated plus maze; NOR, novel object recognition test; L/D box, light/dark box; Tail ST, tail suspension test; ip, intraperitoneally; sc, subcutaneous; im, intramuscular.

is different in men and women (Kendler and Gardner, 2014). Whether testosterone plays a major role in the sex differences in depression is unclear, but a number of studies indicate that it can affect the mood of depressive patients as well as healthy probands (Mchenry et al., 2014). Nevertheless, it is only one of many biological factors potentially responsible for the sex differences in depression. These were reviewed recently (Altemus et al., 2014). Observational studies on older men revealed that their depressive symptoms are associated with low plasma testosterone (Joshi et al., 2010). Low testosterone and depressive symptoms are both associated with the risk of falls, which are important for life expectancy in the elderly (Kurita et al., 2014). Similarly, in women testosterone concentrations are lower in depressive patients when compared to healthy controls (Kumsar et al., 2014). However, standard antidepressant treatment leads to normalization of testosterone. This suggests that the causality could be different than predicted—depression lowers testosterone. On the other hand, in both men and women, testosterone supplementation leads to improvement of depressive symptoms (Pope et al., 2003; Miller et al., 2009). However, not all interventional studies confirmed the anti-depressant effect of testosterone. At least in one published randomized controlled trial, the effects of testosterone were comparable to placebo effects (Seidman et al., 2001). Similarly, not all observational studies show a consistent picture. At least in one small study, depressive women had higher testosterone (Weber et al., 2000). When publication bias and the high intra- and inter-individual variability of testosterone are taken into account, these small negative or contradictory studies could be even more important. The meta-analyses of the published studies are also to be taken into account. In a meta-analysis of the effects of testosterone on depression, the anti-depressant effect was positive, at least in patients suffering from hypogonadism (Zarrour et al., 2009). The biology of the association between testosterone and depression has been reviewed recently (Mchenry et al., 2014). In an animal model of aging the associated depressive-like behavior correlated with lower testosterone (Egashira et al., 2010). Aged mice of both sexes benefited from testosterone supplementation. In the forced swim test the aged mice treated with testosterone or its metabolites spent less time immobile suggesting that the antidepressant effect of testosterone is mediated via several pathways including the androgen and the estrogen receptor (Frye and Walf, 2009). Another experiment on intact rats revealed that the effect of testosterone on depression is dose-dependent (Buddenberg et al., 2009). Interestingly, similar experiment on gonadectomized rats showed that the testosterone metabolite—3-alpha androstanediol, but not testosterone reverted the depression induced by gonadectomy (Frye et al., 2010). Selected animal experiments on the effects of testosterone on depression are compared in **Table 2**.

SPATIAL ABILITIES

Spatial cognitive abilities as well as general cognition and memory decline with aging together with the testosterone levels. During the productive ages and even in early adulthood, men generally outperform women in spatial abilities (Linn and Petersen, 1985). Especially, mental rotation shows a clear sex difference in favor of men. Not surprisingly, observational studies have focused on the association between testosterone and spatial abilities. Some studies have found a positive relationship between testosterone and mental rotation in men (Silverman et al., 1999). Error rate as well as the reaction time negatively correlated with testosterone (Hooven et al., 2004). However, it is not only the actual concentration of testosterone that is studied in relation to spatial performance. Prenatal testosterone and its proxy—the finger length ratio (second to fourth digit) seem to have a stronger association with figure-disembedding and targeting, as additional spatial abilities (Falter et al., 2006). In this study, mental rotation was affected only by sex. In another study, actual testosterone was not associated with spatial abilities, but prenatal testosterone correlated positively with spatial abilities in women (Kempel et al., 2005). In line with these findings is the lack of an association between actual salivary testosterone levels and mental rotation in men and women (Puts et al., 2010). However, in a large observational study analyzing spatial abilities in adult men from various age categories, low testosterone was associated with better spatial visualization (Yonker et al., 2006). In a very interesting study, it was found that in men, the pubertal concentrations of testosterone are negatively associated with mental rotation in the adulthood (Vuoksimaa et al., 2012). In the same paper, the comparison of twins is reported. The twin with higher testosterone scored worse in the mental rotation tests. The results are contradictory, but may depend on the test used for the assessment of spatial abilities. When virtual Morris water maze was used, a positive correlation between testosterone and spatial navigation was found in women, but not in men (Burkitt et al., 2007). The size of the corpus callosum seems to add complexity in the relationship between spatial abilities and testosterone (Karadi et al., 2006). This might be one of the causes for negative findings in studies where some of the determinants are missing (Kubranska et al., 2014). Another cause is likely the selection of the tested population. In gifted children, a negative correlation between salivary testosterone and spatial abilities was found (Ostatnikova et al., 1996). In Chinese men, the accuracy in mental rotation tests was comparable to Americans, but the reaction times were longer indicating that cultural differences could add to the variability of published results (Yang et al., 2007). Last but not least, genetic factors likely modulate the effect of testosterone. We have previously shown that at least in gifted boys, genetic polymorphisms influencing testosterone metabolism affect also its relationship to mental rotation (Celec et al., 2009, 2013). Especially, the CAG short tandem repeat in the exon 1 of the androgen receptor gene seems to be important for the action of testosterone and its metabolites (Nowak et al., 2014). Despite all complexity, the current picture indicates that the association between testosterone and spatial abilities is curvilinear and sex-dependent. In women higher testosterone is associated with better mental rotation, in men lower testosterone is associated with better spatial abilities. This seems to be true both for actual testosterone (Moffat and Hampson, 1996) and for prenatal testosterone (Grimshaw et al., 1995). Supplementation of testosterone in older men results in improvement of spatial abilities, but it is accompanied with changes in estradiol metabolism and it is likely that this interferes with modifications of spatial abilities (Janowsky et al., 1994). Even in rats, testosterone administration affects the strategy of

Table 2 | Selected animal studies analyzing the relationship between testosterone and depression.

Species	Strain	Gender	Timing	Intervention	Maze	Mechanism	Outcome	References
Mus musculus	129:C57BL/6J	Females	Perinatal	Testosterone administered sc at 1st postnatal day; OVX at 28th postnatal day with testosterone capsule insertion	Tail ST	Organizational or activational effect	↑ immobility time in intact and all testosterone groups (depressive effect of testosterone)	Goel and Bale, 2008
Rattus norwegicus	Wistar	Males	Young adults	Testosterone application 15 min before testing in 3 doses (1, 2, 4 mg/kg)	FST	Observational	Failed to confirm main group effect of TST for immobility; 2 and 4 mg/kg groups spent less time immobile during 2nd trial (antidepressant effect)	Buddenberg et al., 2009
Mus musculus	SAMP10 SAMR1	Males	28–34 weeks	No intervention	Tail ST	Observation	SAMP10 prolongation in immobility time of tail suspension in comparison to SAMR1; SAMP10 showed lower TST levels but not DHEA; SAMR1 and SAMP10 showed significant correlation of TST and immobility; r= −0,667	Egashira et al., 2010
Mus musculus	C57/BL6	Males and Females	24 months (range 20–28)	Intact aged mice; 1 h before testing 1 mg/kg of TST, E2, DHT, or 3-alpha diol administered sc	FST	Observational	Main effect of sex and androgen for immobility; Aged male ↑ time immobile compared to other male groups; Aged female mice were less immobile than aged male mice (antidepressive effect of androgens and E2)	Frye and Walf, 2009
Rattus norwegicus	Sprague-Dawley	Young adults	Males and females	Gonadectomy in adulthood; Females masculinized by TP at PD 1	Learned helplessness Avoidance test Tested in adulthood	Observational/ mechanism either TST or E2 organization/ activation effect	All Males not able to learn to escape stress during training; all females learned to escape; TST and metabolites from periphery did not influence depressive behavior through organizational effect	Dalla et al., 2008
Mus musculus	C17/BL6	Males	8–10 weeks	ArKO mice and null KO	FST	Mechanism—through AR receptor?	ArKO did not differ in FST; ArKO exhibited normal levels of motor activity, anxiety and depression; CUMS had no effect	Dalla et al., 2005

(Continued)

Table 2 | Continued

Species	Strain	Gender	Timing	Intervention	Maze	Mechanism	Outcome	References
Rattus norwegicus	Wistar	Males and females	Prepubertal and young adult males and females in estrus	No intervention	FST	Observation	Prepubertal rats of both sex increased immobility, adult males higher immobility than adult females; (depressive effect of testosterone)	Martinez-Mota et al., 2011
Rattus norwegicus	Wistar	Males and females	Young adults and 12–15 months adult rats	Gonadectomy in younger TST containing pellets in older animals	Anhedonic test CUMS		TST before CUMS prevented anhedonia in older rats; in young, gonadectomy did not increase vulnerability to anhedonia	Herrera-Perez et al., 2012
Rattus norwegicus	Sprague-Dawley	Males and females	Young adults	Gonadectomy, with pellet of TST or imipramine	Anehodonia test Novelty induced hypophagia	Observational	Testosterone had anxiolytic and antidepressant effect in males but not in OVX females; same effect as imipramine	Carrier and Kabbaj, 2012

FST, forced swim test; CUMS, chronic unpredictable mild stress; Tail ST, tail suspension test; TST, testosterone; TP, testosterone propionate; E2, estradiol; DHEA, dehydroepiandrosterone; SAMP, senescent prone mice; SAMR, senescent resistant mice; ArKO, androgen receptor knock-out; PD, postnatal day; sc, subcutaneously.

the animals in spatial tasks (Spritzer et al., 2013). However, the interaction between testosterone and mental rotation tests is bidirectional. It has been shown that mental rotation testing affects testosterone, at least in women (Durdiakova et al., 2012). In **Table 3**, published experimental data on the effects of testosterone on spatial abilities are summarized.

MEMORY

Women have better verbal memory, while men have an advantage in visual-spatial memory (Lewin et al., 2001). Especially, the difference in spatial memory has been studied in detail (Shah et al., 2013). In a meta-analysis of animal experiments using radial and water mazes, it has been confirmed that males outperform females in spatial memory tasks (Jonasson, 2005). The positive effect of testosterone on memory was, however, well documented in both sexes. Numerous clinical studies in postmenopausal women and men in the andropause showed improvements of learning and memory after testosterone supplementation. Even a short 6-week testosterone treatment resulted in improved spatial and verbal memory of older men (Cherrier et al., 2001). Testosterone has even showed a positive effect on spatial and verbal memory in Alzheimer disease patients (Cherrier et al., 2005). In young women, a single dose of testosterone improved spatial memory (Postma et al., 2000). However, the mechanism of action is unclear, as testosterone is now rather considered as a precursor than as a final hormone. In contrast to some animal experiments, observational studies in elderly men showed that lower testosterone, especially its free fraction was associated with worse visual-spatial memory (Moffat et al., 2002). This might be related to the tasks used, as the testosterone levels in men are related to the learning strategies, especially for spatial memory (Choi and Silverman, 2002). The results are, however, inconsistent. In a study analyzing the effects of a single testosterone injection on elderly men the treatment caused a worsening of verbal memory (Wolf et al., 2000). Similarly, biweekly injections of testosterone during 90 days resulted in memory decline (Maki et al., 2007). In addition, patients with prostate cancer that need hormonal castration via androgen deprivation therapy had worse verbal memory than heathy controls. Interestingly, estradiol—the estrogen metabolite of testosterone reversed the negative effects of androgen deprivation (Beer et al., 2006). Similar findings were found in elderly men and women where estradiol slowed down the age-related memory decline (Carlson and Sherwin, 2000). It seems that the effect of testosterone is dose-dependent and could be curvilinear even within sexes. At least in men, it has been demonstrated that moderate dosing resulted in improved memory, but not low and very high increases of testosterone (Cherrier et al., 2007). Similar results were found in adult male rats where only moderate testosterone doses resulted in spatial memory improvements (Spritzer et al., 2011). A relatively high dose of testosterone had no effect on memory or other analyzed behavioral measures in postmenopausal women in a well-designed and large study (Kocoska-Maras et al., 2011). In another study, women with surgically induced menopause received testosterone or placebo in addition to estrogen supplementation. Testosterone in this case worsened the verbal memory (Moller et al., 2010). A smaller, but longer study on postmenopausal women showed

Table 3 | Selected animal studies analyzing the relationship between testosterone and spatial abilities.

Species	Strain	Gender	Timing	Intervention	Maze	Mechanism	Outcome	References
Rattus norwegicus	Sprague-Dawley	Males and females	Young adults	Gonadectomy or sham with or without testosterone capsule implantation (25 mg TST)	12-arm radial maze	Observational/ pharmacological study	Intact males performed better in working memory tasks than intact females; Castration impaired working memory but not reference memory performance in males	Gibbs and Johnson, 2008
Rattus norwegicus	Wistar	Males	Young adults	Gonadectomy, TP 0.0625–1.0 mg sc or oil each day	8-radial maze, MWM	Observation	No effect of TST in 8-radial maze, and in MWM in terms of main effect of treatment	Spritzer et al., 2011
Rattus norwegicus	Wistar	Males	Young adults	CA1 injections of TST or DMSO or intact	MWM	Observation	TST increased latency times (worsened memory) by 200% to intact and 100% in DMSO group	Emamian et al., 2010
Rattus norwegicus	Wistar	Males	Intact/sham castrated/dolesce; If castrated then at PD 22, but trained at PD 28,35,45, and 60	TST or oil applied between PD 30-37	MWM	Observation	Pre-pubertal castration improved spatial ability in mid dolescence, but no effect in adults	Moradpour et al., 2013
Rattus norwegicus	Sprague-Dawley	Males	Young adults	GDX with TST, DHT, E2 or oil capsule sc	Object location memory	Observation	GDX impaired spatial memory; TST, E2 and DHT reversed effect	Mcconnell et al., 2012

GDX, gonadectomy; TST, testosterone; DHT, dihydrotestoterone; E2, estradiol; TP, testosterone propionate; MWM, Morris water maze; PD, postnatal day; sc, subcutaneously.

the complete opposite—improvement of verbal memory after testosterone treatment (Davison et al., 2011). Similarly to other behavioral measures memory will be influenced also by prenatal concentrations of testosterone (Bull et al., 2010). This effect might be mediated by the organizational effect of testosterone on brain structures such as amygdala or hippocampus (Ackermann et al., 2012). It has been shown that prenatal and neonatal testosterone affects stress coping and the effects of stress on learning abilities, at least in rodents (Shors and Miesegaes, 2002). Of course, genetic factors might also play a role. At least in one study the APOE genotype interacts with testosterone regarding the influence on age-related cognitive decline (Panizzon et al., 2014). More such studies can be expected in the near future.

Animal experiments help us to uncover the molecular and physiological mechanisms behind the phenotype correlations seen in human studies. The organizational effect of testosterone on the hippocampus, the major memory structure in the brain has been described a long time ago in rats using various mazes (Roof and Havens, 1992; Roof, 1993). In birds, evidence exists for a low testosterone period needed during the development of brain functions such as vocal memory (Korsia and Bottjer, 1991). In aged rats, an important experiment showed that the positive effect was found only when testosterone was administered. The testosterone metabolite, dihydrotestosterone, which cannot be metabolized to estradiol did not showed this effect (Bimonte-Nelson et al., 2003). This indicates that the effect of testosterone on memory is mediated by estradiol and the effect of aromatase which converts testosterone to estradiol. However, in male deer mice it has been shown that aging but not testosterone affects memory (Perrot-Sinal et al., 1998). Testosterone might rather increase synaptic plasticity as shown in rats (Schulz and Korz, 2010). Increased plasticity, however, only enables improved memory. But whether the potential is used depends on other factors including environment and timing and form of learning. Another advantage of animal experiments is the possibility to surgically localize the administration of testosterone into specific brain structures, which is ethically not possible in humans. Such studies showed that in adult male rats administration of any dose of testosterone or the androgen receptor blocker flutamide resulted in worsening of spatial memory (Naghdi et al., 2001). Similar injections of flutamide into amygdala had no effect on spatial memory, but testosterone negatively affected spatial memory and learning (Naghdi et al., 2003). When histological analyses were conducted, it was found that the intrahippocampal injections of testosterone led to an increase in the number of astrocytes in the target area (Emamian et al., 2010). Co-administration of a protein kinase AII inhibitor resulted in a synergistic negative effect on spatial memory (Khorshidahmad et al., 2012). Interestingly, the injection of anastrozole—an aromatase inhibitor resulted in improvement of spatial learning and memory tested in the Morris water maze (Moradpour et al., 2006). This further confirms that the negative effect of testosterone on memory is localized to hippocampus and is mediated by estradiol. When dihydrotestosterone—the androgen metabolite of testosterone was injected into the CA1 region of the hippocampus, spatial memory was improved (Babanejad et al., 2012). Testosterone has very likely an important role in the physiology of brain functions, but it might also be useful in some pathologies. In castrated rats, testosterone was able to reverse the ethanol-induced memory deficit (Khalil et al., 2005). In diabetic rats, the memory impairment was partially reversed by testosterone administration as well (Nayebi et al., 2014). An experiment in mice contributed to the growing list of confounding variables with the length of the photoperiod. Castration and supplementation with testosterone had no effect when the photoperiod was long (16 h of light per day). On the contrary, in mice housed with a short photoperiod (8 h of light per day), the effects on spatial memory were clearly seen (Pyter et al., 2006). A selection of the numerous animal experiments focusing on testosterone and memory are presented in **Table 4**.

FUNCTIONAL MAGNETIC RESONANCE IMAGING IN HUMANS

Functional magnetic resonance imaging (fMRI) is a neuroimaging procedure that uses MRI technology for measuring the brain activity. The principle lies in detection of associated changes in blood flow and is useful in mapping the brain functional areas (Hofer et al., 2013). Several studies were performed using human volunteers for spatial tasks, memory as well as mood disorders/traits.

As for spatial tasks and mental rotation, the fMRI data are valuably consistent. In line with the previous studies, the males outperformed females in spatial tasks. Additionally, the fMRI showed stronger activation of left inferior parietal lobe in males compared to females. Also, the testosterone levels correlated with activation levels during mental rotation task in males. In females, the early follicular and midluteal phases were associated with better outcome and higher estradiol concentrations (Schoning et al., 2007). Likewise, a study of van Hemmen et al. confirmed previously reported sex differences in neural activation during mental rotation. Moreover, participants with complete androgen insensitivity syndrome presented with female-like neural activation pattern in the parietal lobe, indicating that gonadal hormone exposure rather than genetic sex itself plays role in brain functions (Van Hemmen et al., 2014). The menstrual cycle and thus the involvement of sex hormones, including testosterone, in spatial abilities was further confirmed by Pletzer et al. In their study, error rates linked with deactivation of inferior parietal lobes and prefrontal lobes were higher during luteal phase for verbal tasks, while in the follicular phase, spatial abilities in females were confirmed (Pletzer et al., 2011).

ISSUES

PSYCHOMETRIC TESTS

The analysis of behavior is not as straightforward as biochemical and molecular methods. Several alternatives exist for testing any brain function. However, the tests are variable and it is only a consensus, which can or should be used. The same applies to mazes used for the assessment of animal behavior. Even for the widely used Morris water maze several alternatives exist and numerous different parameters are used in the particular studies. An experiment showed that testosterone does not affect some of the measures analyzed in the water maze, but does affect other measures such as spatial working memory retention (Sandstrom et al., 2006).

Table 4 | Selected animal studies analyzing the relationship between testosterone and memory.

Main objective	Method	Result	References
Whether long term TST restoration improves vasopressin innervations and spatial learning memory	Three groups of male rats by age (young, middle aged and senescent) treated with TST or sham in MWM	TST treatment did not improve spatial learning or retention of spatial information. Aged rats performed worse than young	Goudsmit et al., 1990
If testosterone improves spatial abilities in adulthood, when administered neonatally	Testosterone propionate applied to neonatal rats; males and females, tested in adulthood	TST increased performance in control group males outperformed females, in TST group the pattern was reversed	Roof, 1993
Spatial learning and circulatory levels of testosterone in plasma	Males and females of Meadow voles according to TST and E levels underwent MWM	Male superiority was evident only with high estradiol female group, no difference between high and low TST groups	Galea et al., 1995
Whether TST treatment neonatally affects spatial leasing in adulthood in gonadectomized rats with frontal cortical lesion	Neonatally gonadectomized rats (females and males) with or without testosterone treatment underwent MWM in adulthood	Lesions at day 7 did not impair spatial learning but gonadectomy or testosterone propionate impaired the learning	Kolb and Stewart, 1995
If chronic administration of anabolic-androgenic steroids improve spatial cognition	Three groups of males supplemented with nandrolone, oil and steroid cocktail for 12 weeks, then MWM	No differences in spatial tasks in any of the treated groups	Clark et al., 1995
Investigate the effect of reproductive status on spatial learning in several reproductive stages	Meadow voles and deer mice tested in MWM either in breeding or non-breeding stage in adulthood or as juvenile	Better performance of males when females in estrus, otherwise no difference; High-E females performed worse than low-E females or males. No difference until adulthood	Galea et al., 1996
How testosterone supplementation influences spatial learning after frontal lesions in both sexes	Eight groups in experiment, females (treated with testosterone or vehicle) and males (gonadectomized or sham), all groups moreover either with frontal cortex lesion or sham	No difference of sex or hormonal manipulation, but males with lesion performed better than females with lesion	Forgie and Kolb, 1998
Spatial learning in male deer mice in relation to age	Four groups of deer mice divided by age performed in MWM. Mice were divided according to breeding state	Young and young breeding mice performed better (higher TST) than old and even young non-breeding (lower TST) mice	Perrot-Sinal et al., 1998
If prenatal androgen and estrogen affects adult spatial learning	TST and DHT females, EB females and flutamide males with prenatally (day 16) treatment were tested in adulthood in MWM	TST and EB sex differences observed in MWM as a prenatal component	Isgor and Sengelaub, 1998
If there is difference in spatial memory in females through oestrus	Male and female rats tested in MWM during several estrus cycles	No overall sex difference I retention spatial memory, females latency in estrus was longer	Healy et al., 1999
If androgen exposure impairs cognitive functions in SHR	Implantation of TST neonatally, and tested in MWM on 45th day	Androgen impaired spatial memory in SHR	King et al., 2000
Testosterone and flutamide effect on spatial performance	Intrahippocampal administration of TST or flutamide 30 min prior testing in MWM	Increased latencies in both treated groups, dose dependent	Naghdi et al., 2001
Role of sex steroids in apoE4 induced cognitive impairment	Mice expressing human apoE4 or E3 treated with testosterone and tested in MWM	Treatment improved memory deficits in apoE4 females	Raber et al., 2002

(Continued)

Table 4 | Continued

Main objective	Method	Result	References
Developmental androgen sensitivity of CA3 area and spatial performance	Neonatally TST or ovariectomized females and TST castrated and TST treated or not performed in MWM during adulthood	High androgen groups did better than low androgen groups	Isgor and Sengelaub, 2003
If testosterone and flutamide in amygdale affect the spatial abilities	Testosterone or flutamide administered into amygdale 30 min prior testing in MWM	Testosterone dose dependent increase in latency times, no effect of flutamide	Naghdi et al., 2003
If testosterone improves cognition in older rats	Young and old TST or DHT treated rats underwent water radial maze	TST (but not DHT) improved spatial memory in older rats	Bimonte-Nelson et al., 2003
If testosterone improves spatial cognition in female rats	TST, DHT, Estradiol or control ovariectomized female rats tested after 48 h in MWM	Estradiol impaired spatial acquisition. TST and DHT without effect	Frick et al., 2004
Compare wild-types and testicular feminization mutation rats	Tfm and control male rats and heterozygote females performed in water maze	Males control outperformed females; Tfm showed intermediate performance	Jones and Watson, 2005
Evaluate effects of testosterone and ethanol on spatial cognition	Male rats castrates with ethanol, testosterone or both performed in water maze	Ethanol induced deficits in spatial cognition; testosterone reversed this effect	Khalil et al., 2005
Role of testicular hormones on spatial abilities	Castrated and intact males performed in MWM and delayed-matching-to-place MWM	Castration impaired working memory retention, reversed by exogenous testosterone	Sandstrom et al., 2006
If photoperiod affects spatial learning through testosterone reduction	Mice either in 16 or 8 h daylight for 14 weeks performed in MWM after castration/sham/castration+ testosterone	Castrated with testosterone short day mice performed better to other short day mice, in long day no differences	Pyter et al., 2006
Evaluate effect of TST, estrogen and anastrazol on spatial abilities	CA1 cannulation of adult male rats with various dosages of testosterone, estradiol, anastrazol or DMSO	TST and estradiol impaired spatial learning, anastrazol improved it	Moradpour et al., 2006
If castration of males affects the spatial memory	Castrated and sham male rats performed in MWM	No differences between groups	Spritzer et al., 2008
Spatial learning and TST	Castrated and intact male rats, rats cannulated into right or left hippocampus castrated or not did spatial task in MWM	Castration did not affect learning	Mohaddes et al., 2009
Investigate effects of TST metabolites on spatial performance	Male rats subjected to orchiectomy and capsule with TST metabolites implanted did spatial tasks in MWM	3-alpha and 3-beta-diols enhanced spatial cognition	Osborne et al., 2009
Enhancement of aged female rats by androgen supplementation	Old mice with implanted TST or DHT or empty capsules performed in MWM after 6 weeks	TST improved spatial cognition, DHT did not	Benice and Raber, 2009

TST, testosterone; DHT, dihydrotestoterone; EB, estradiol benzoate; Tfm, testicular feminization mutation; DMSO, dimethytlsulfoxide; MWM, Morris water maze.

POPULATIONS

One of the major factors that might explain the differences between the results of various studies is the variability of the examined populations. As mentioned above, the cultural differences, sex and age have all been shown to impact the physiological effects of testosterone. In animal experiments, chosen species and the particular strain is also of importance. Looking at the studies in non-human primates in contrast to the majority of rodent studies the results are mostly negative. For example, testosterone manipulations in rhesus monkeys did not alter their working and

reference memory, although emotional processing was affected. Indeed, the treatment for testosterone might have not last long enough to affect the cognition (Kelly et al., 2014). Other possible explanations might be due to low number of animals included, but also to physiological differences including body size and the concluding testosterone kinetics (King et al., 2012). Specific behavioral tests might also be responsible for the differences observed (Lacreuse et al., 2012).

TESTOSTERONE MEASUREMENT

There are several possibilities as to what kind of biological samples should be used for the testosterone measurement. Plasma, saliva, urine are available and all have some strengths and weaknesses. The simple scheme of free—bioactive fraction of testosterone that should be assessed using salivary testosterone or plasma albumin and sex hormone binding globulin is not correct. Bound testosterone has its effects on target tissues and it is not clear which of the potential biological liquids is robust against technical and biological variability. One of the exotic possibilities is measurement of testosterone in the hair. The concentration in the hair might, however, be relevant as it integrates all the intra-individual variability of testosterone (Dettenborn et al., 2013).

TIMING

Testosterone undergoes several biorhythms. In some studies, even the best-known circadian rhythm is not taken into account. Implants that slowly release testosterone totally ignore daily variations that occur physiologically. Other rhythms such as infradian cycles are completely forgotten when experiments are designed. But beyond cyclic variations, testosterone undergoes chaotic temporary changes that are usually described as noise. Although such research is lacking, it might be that it has some physiological role similarly to heart rhythm variability. In addition, the timing of behavioral analyses is of importance. While within 30 min after administration, non-genomic effects are important, later genomic effects are expected to be the major mediator. But this does not have to be true. Even later, the non-genomic effects are active in parallel with the gene expression changes. Only the study of the particular effects is more and more complicated, especially due to the complex kinetics of testosterone and the complex abilities being tested as proved in a focused experiment (Hawley et al., 2013). Additionally, physiological and also behavioral functions are exerted on a rhythmic basis. Timing the behavioral tests for light phase, while rodents usually are active during night can represent a major problem in animal behavior testing. Moreover, the central circadian clock is located in the suprachiasmatic nucleus of the hypothalamus and it receives signals directly from photoreceptors. GABA is thought to play a major role in coordinating the synchronized firing of suprachiasmatic neurons (Urbanski, 2011). However, steroid hormones may also exert their nongenomic function through GABA receptors. Disrupting the GABAergic system by untimed testosterone application, may be one other reason for controversy results in behavioral analysis. Alternatively, aging is strongly related to decline of circulating sex hormones, disrupting thus also circadian rhythms and leading to impaired sleep or cognitive functioning (Urbanski et al., 2014). Restoring natural circulating hormone pattern in older but also in younger animals could possibly lead also to more comprehensive results of sex hormones and behavior studies.

ADMINISTRATION ROUTE

In most studies, testosterone is injected via i.p. or i.m. injections, but there are indices that to study the effects of testosterone on brain functions, the steroid has to be injected directly into the target brain structure. At least in one experiment directly comparing peripheral administration and intrahippocampal injections of testosterone it was shown that the peripheral route had no effect on learning and memory while central injections were effective (Harooni et al., 2008).

TYPE OF TESTOSTERONE USED

Testosterone in the experiments is sometimes used as butyrate, decanoate, undecanoate etc. These pharmacological forms have, however, variable kinetics and might therefore have also variable effects, especially in the brain, where the kinetics is of special importance (Filova et al., 2012). Dosing of testosterone seems to be of enormous importance. It varies between the experiments widely and should always be taken into account when evaluating the results. In experiments, moderate, but not very low or very high doses of testosterone had some effect on behavioral measures such as memory (Spritzer et al., 2011).

The effect of testosterone is influenced by several factors, but only some of them are known. These include genetic polymorphisms related to testosterone metabolism or other pathways related to cognitive functioning (Panizzon et al., 2014). Next generation sequencing and lower prices of genotyping will enable detailed studies focusing on the genetic factors and especially on the complex interactions between genetic, endocrine and other environmental factors.

METABOLISM

Testosterone is currently seen more as a precursor of hormones. In most target tissues, testosterone is converted into metabolites such as dihydrotestosterone—a more potent androgen receptor ligand. The enzyme aromatase, on the other hand, can metabolize testosterone into estradiol—a ligand of the estrogen receptors. Further metabolites are being added to the list. But in general, it is of importance to recognize the role of the target tissue that can convert testosterone to inducers of very different signaling pathways. Without genetic or pharmacologic manipulation it is not possible to distinguish the effects when testosterone itself is administered.

NON-GENOMIC EFFECTS

The metabolism of testosterone makes studying the physiology of testosterone effects on the brain difficult. But the response of target tissues are similarly complex. Testosterone can be recognized by the androgen receptor inducing genomic effects—changes in gene expression. But the same testosterone can induce other signaling pathways that do not require changes in the use of the genomic information. These effects are called non-genomic and are studied for all steroid hormones. When testosterone is injected into the hippocampus together with a protein synthesis inhibitor that prevents genomic effects, spatial memory is improved in

male rats (Naghdi et al., 2005). This points toward the possibility that non-genomic effects can be opposite to the genomic effects. But it also shows that doing such experiments and interpreting their results is difficult. Inhibition of protein synthesis is of course not specific. An alternative is to analyze the behavior rapidly after testosterone injection, as it take roughly 30 min to induce gene expression changes. But the kinetics of testosterone *in vivo* complicates the interpretation. Another option is the co-administration of androgen receptor and estrogen receptor blockers.

CONCLUSION AND FUTURE OUTLOOK

While fMRI results bring interesting data and knowledge on behavioral traits and spatial abilities in relation to testosterone levels and sex differences, the result obtained can show only association or correlation but not causal relationship of testosterone effect on behavior. Nevertheless, also according to the numerous published studies and animal experiments, testosterone seems to affect brain functions. The high number of relevant publications also indicates that it is a hot topic of interest. However, quantity is not quality and currently, despite numerous publications it is very difficult to conclude how testosterone affects cognitions and emotions. Most of the published literature agrees on the fact that testosterone is anxiolytic, anti-depressant and improves spatial abilities. But this picture is oversimplified. Many variables add to the complex interactions between testosterone and the brain. Memory, both, verbal and spatial, is a good example. Age, sex, current endocrine status, but also the timing of testosterone analysis or administration, status of the target tissues and several other factors influence the outcome of observational or interventional studies. It is, thus, clear that small studies can only describe a very small window of the whole complex physiology. Analyzing testosterone concentrations, choosing appropriate doses and pharmacological forms is difficult enough. The psychometrics behind behavioral tests in animal experiments and behind psychological tests in human studies is, nevertheless, lacking. Standardization in this area would surely improve our understanding of the neuroendocrinology of testosterone. More systematic research using the whole spectrum of available tools and looking at the various physiological aspects is needed. However, to be able to publish such research, journals should accept manuscripts based on the design and not on the results. Otherwise, the publication bias that is obvious in the so far published literature will continue to be a big issue. Many researchers in this field complain about negative results that are very difficult to publish in the relevant journals. The number of such unpublished observations and experiments is unknown. But based on our humble experience, the negative results will probably be more common than the published positive ones. And if the contradictory published findings are added, the picture gets even more confusing. Large systematic research projects with more cooperation between the most productive research teams is definitely needed.

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Testosterone increases circulating dehydroepiandrosterone sulfate levels in the male rhesus macaque

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The adrenal steroid dehydroepiandrosterone (DHEA) and its sulfate (DHEAS) are two of the most abundant hormones in the human circulation. Furthermore, they are released in a circadian pattern and show a marked age-associated decline. Adult levels of DHEA and DHEAS are significantly higher in males than in females, but the reason for this sexual dimorphism is unclear. In the present study, we administered supplementary androgens [DHEA, testosterone and 5α-dihydrotestosterone (DHT)] to aged male rhesus macaques (*Macaca mulatta*). While this paradigm increased circulating DHEAS immediately after DHEA administration, an increase was also observed following either testosterone or DHT administration, resulting in hormonal profiles resembling levels observed in young males in terms of both amplitude and circadian pattern. This stimulatory effect was limited to DHEAS, as an increase in circulating cortisol was not observed. Taken together, these data demonstrate an influence of the hypothalamo-pituitary–testicular axis on adrenal function in males, possibly by sensitizing the zona reticularis to the stimulating action of adrenocorticopic hormone. This represents a plausible mechanism to explain sex differences in circulating DHEA and DHEAS levels, and may have important implications in the development of hormone therapies designed for elderly men and women.

Keywords: adrenal gland, aging, androgen, dehydroepiandrosterone, non-human primate, testosterone

INTRODUCTION

Levels of the adrenal steroid dehydroepiandrosterone (DHEA) and its sulfate (DHEAS; collectively referred to as DHEA/S) differ between males and females, yet the underlying cause for this sexual dimorphism is unknown (1). It is clear, however, that the gross, cellular, and molecular adrenal structure of males and females is essentially similar, suggesting that some factor outside the adrenal gland is responsible for the greater secretion of DHEA/S in males (2). Given that the sex differences in DHEA/S are most pronounced during early adulthood, it is plausible that the different levels of sex-steroid hormones between adult males and females may play a contributing role. In fact, previous work in perimenopausal women and macaque models of menopause has shown that decreasing levels of estrogens correspond to increases in DHEAS (3–5), and estrogen replacement paradigms decrease DHEA/S (5, 6) at a rate greater than seen in normal aging without estrogen supplementation. As DHEA/S is a precursor to estradiol (7), this may reflect a compensatory interaction between the hypothalamic–pituitary–gonadal (HPG) and hypothalamic–pituitary–adrenal axes. On the other hand, males have higher circulating levels of DHEAS than do females, despite having substantially higher levels of testosterone (T). This suggests that an inverse relationship between adrenal and gonadal steroids exists in females, whereas a positive causal relationship may exist in males.

The hormone systems discussed so far not only have the potential for interacting with each other, but they also show significant age effects. In both sexes, DHEA/S declines gradually and consistently starting in early adulthood, with levels reaching roughly 40% of their peak by the middle age and continuing to decline thereafter (8, 9). In females, menopause is associated with a sudden decline in estrogen (10, 11), while males show a less precipitous decline in testosterone during aging. The situation is further complicated by therapeutic hormonal supplementation in the elderly, which can alter circulating sex-steroid hormone levels, though not necessarily in the most appropriate physiological manner. Furthermore, in the USA DHEA is sold as a food supplement, requiring no prescription, and so is widely used by the elderly for self-medication. Thus, to fully understand the physiological impact of hormone changes with age the interactions between the HPG and HPA axes require further investigation under carefully controlled experimental conditions, something that can be achieved more readily using non-human primates such as the rhesus macaque.

Physiological replacement of steroids may protect against many negative aspects of aging, but it is first important to understand the differences between males and females and how these systems interact to determine the safest and most effect form of hormone therapy (HT). We used the rhesus macaque, a large diurnal primate with endocrine physiology very similar to that of humans, to investigate the interactions between DHEA/S and testosterone. Because we recently observed novel interactions in a combined androgen supplementation paradigm (12, 13), we have expanded on this previous research to formulate a working hypothesis for adrenal and gonadal interactions. Our results indicate a strong

influence of testosterone on circulating levels of DHEAS, possibly explaining the drastic differences between male and female levels of the hormone.

MATERIALS AND METHODS

ANIMALS

The study used adult rhesus macaques (*Macaca mulatta*), and was approved by the OHSU Institutional Animal Care and Use Committee. The animals were cared for by the Division of Comparative Medicine at the Oregon National Primate Research Center (ONPRC) in accordance with the *National Research Council's Guide for the Care and Use of Laboratory Animals*. They were caged singly indoors under controlled environmental conditions: 24°C temperature; 12-h light, 12-h dark photoperiods (lights on at 0700 h). Monkey chow was provided at 0800 and 1500 h and was supplemented with fresh fruit and vegetables; fresh drinking water was available *ad libitum*.

In Experiment 1, ten adult (11–12 years) male and eight adult (11–12 years) female rhesus macaques were used to assess sex differences in circulating DHEAS levels. In Experiment 2, four young adult (7–12 years) and four old (21–26 years) males were used to examine age-related changes in circulating DHEAS levels; five additional old males were used to evaluate the impact of androgen supplementation on DHEAS.

REMOTE BLOOD SAMPLING

Cortisol and DHEA/S both demonstrate a circadian pattern of release (14–16). Therefore, to gain meaningful insights regarding sex-related or age-related hormone differences it was necessary to collect blood samples from each animal serially across an entire 24-h period. To achieve this with minimal disruption of the animals, each monkey was surgically implanted with a subclavian vein catheter, leading to a remote blood sampling system in an adjacent room, as previously described (17). Blood samples were collected into EDTA-coated borosilicate glass tubes every hour for a complete 24-h cycle. The samples were centrifuged at 4°C, and the plasma was stored at −20°C until assay for cortisol and DHEAS. Cortisol was assayed using electrochemiluminescence using the Elecsys 2010 Platform (Roche Diagnostics, Indianapolis, IN, USA). DHEAS was assayed using radioimmunoassay with a highly specific antibody for DHEAS-17-(O-carboxymethyl)oxime-BSA (Endocrine Services, Tarzana, CA, USA) and [^{3}H]DHEAS (SA, 22 Ci/mmol). Intra- and interassay coefficients of variation were less than 10% for each assay and the assay detection limits were 3 ng/ml. The 24-h serial blood sampling procedure was performed once on each animal, except for the old androgen-supplemented animals, which were re-sampled after each of the 5-day androgen supplementation tests, performed approximately 1 month apart (**Table 1**). Data were analyzed using a repeated-measures ANOVA with time as a within-subjects factor and group (young, old baseline, old supplemented) as a between-subjects factor. When the assumption of sphericity was not met, a Greenhouse-Geisser correction was implemented.

STEROID SUPPLEMENTATION

Animals were treated with hormones for 5 days prior to each blood sampling session to allow steroid levels to equilibrate.

Table 1 | Steroid supplementation paradigms.

	Time of hormone administration		
Experiment	**0700 h**	**1000 h**	**1900 h**
Baseline	–	–	–
1	DHEA (0.04 mg/kg)	DHEA (0.04 mg/kg)	T (12 mg/kg)
2	DHEA (0.10 mg/kg)	DHEA (0.05 mg/kg)	–
3	DHEA (0.10 mg/kg)	DHEA (0.05 mg/kg)	DHT (5–10 mg/kg)
4	T (12 mg/kg)	–	–

The times and doses of the four androgen supplementation paradigms are shown. Due to limitations on blood sampling, not all combinations of hormones were able to be tested at all doses. DHEA and T were dissolved in sesame oil, and were mixed into chocolates for oral supplementation. Animals were monitored to ensure the entire dose was eaten in a timely manner.

DHEA (10 mg/ml; Sigma-Aldrich, St. Louis, MO, USA), testosterone (T, 120 mg/ml; Sigma-Aldrich), and dihydrotestosterone (DHT, 5–10 mg/ml; Sigma-Aldrich) were dissolved in commercial food-grade sesame oil and mixed with melted chocolate, a preferred treat. The steroids were dissolved in sesame oil to reduce their uptake and rapid metabolism by the liver and instead by increasing their absorption through the lymphatic system (18, 19). Chocolates were kept refrigerated at 4°C until the time of administration. Animals were watched at the time of administration to ensure the entire treat was eaten. To isolate potential mechanisms of adrenal androgen interaction, four supplementation paradigms were performed (**Table 1**): (1) DHEA administered at 0700 and 1000 h, T administered at 1900 h; (2) DHEA administered at 0700 and 1000 h; (3) DHEA administered at 0700 and 1000 h, DHT administered at 1900 h; and (4) T administered at 0700 h. These doses were selected based on preliminary experiments that were aimed at replicating the hormone levels observed in young adults; consequently, the doses varied between experiments. To protect animals against excessive blood sampling, it was not feasible to repeat all hormone combinations at all doses in all of the animals. Times were chosen to replicate the endogenous circadian peaks of T and DHEA, while in the last experiment T was administered in the morning to examine a possible role of time of day on the steroid response to T. Animals were monitored at the time of steroid supplementation to ensure each ate the entire treat in a timely manner; if the treat was refused, an equivalent dose was administered via a steroid-soaked cookie or prune. The doses used for each experiment are provided in the respective figures. As shown previously (13), the dose paradigm used in Experiment 1 is sufficient to restore circulating T and DHT to levels seen in young adult male rhesus macaques.

RESULTS

YOUNG ADULT MALE RHESUS MACAQUES SHOW SIGNIFICANTLY HIGHER LEVELS OF DHEAS THAN FEMALES

Cortisol and DHEAS showed well-defined 24-h plasma profiles, both in the males and females (**Figures 1A,B**, respectively). The plasma levels rose gradually during the night and reached a peak in the morning at about the time when the lights came on. Although mean cortisol levels were similar in the two sexes, mean DHEAS

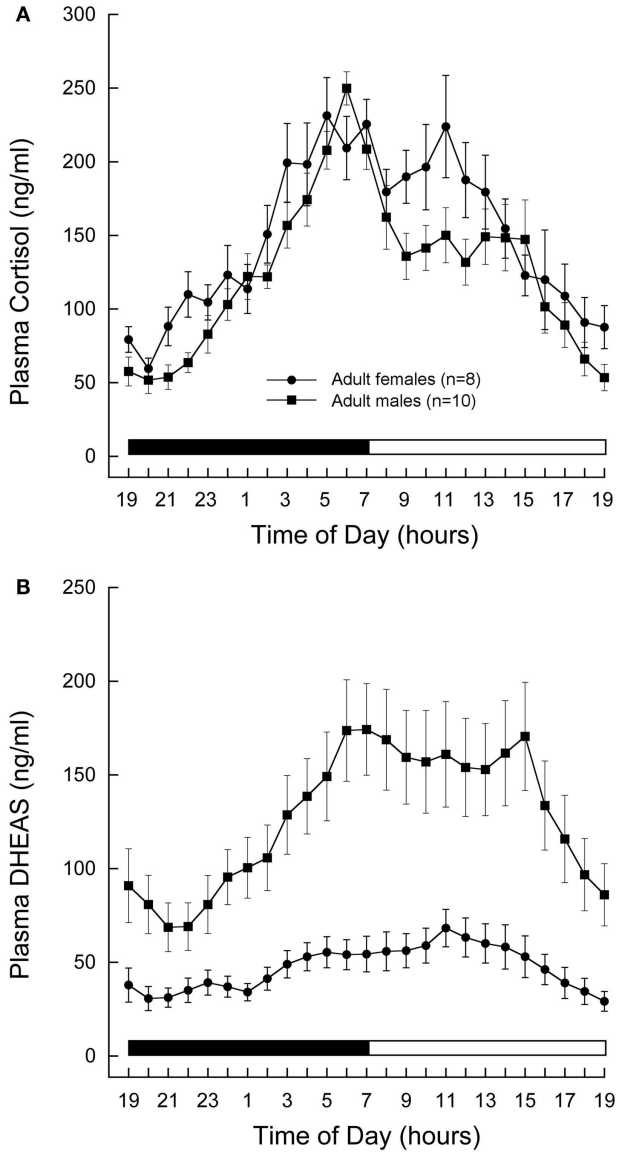

FIGURE 1 | Twenty-four-hour plasma profiles of adrenal steroids in male and female rhesus macaques. Serial blood samples were remotely collected from adult (11–12 years) male ($n = 10$) and female ($n = 8$) animals and assayed for cortisol **(A)** and DHEAS **(B)**. Each data point represents the mean and vertical lines indicate the SEM. Time of day is indicated along the abscissa, and periods of darkness and light are represented by black and white bars, respectively. In both sexes, cortisol and DHEAS levels rose gradually during the night and reached peak levels around the time of lights on in the morning. Mean cortisol levels were similar in the two sexes, whereas mean DHEAS levels were significantly higher in males than in females ($P < 0.01$, Student's t-test.)

levels were significantly higher in males than in females ($P < 0.01$, Student's t-test).

AGING IS ASSOCIATED WITH DECREASED CIRCULATING DHEAS LEVELS

As shown in **Figure 2A**, circulating levels of DHEAS at baseline were significantly lower in aged (open squares) than in young males (open circles). A repeated-measures ANOVA with time as within-subjects factor and group (young and old baseline) as between-subjects factor indicated a significant effect of time ($F = 6.427$, $P < 0.001$), significant effect of group ($F = 21.828$, $P = 0.003$), and a significant group-by-time interaction ($F = 7.146$, $P = 0.001$). Circulating DHEAS was higher in young males at all time points.

TESTOSTERONE ADMINISTRATION IN AGED MALE RHESUS MACAQUES SIGNIFICANTLY INCREASES CIRCULATING DHEAS TO LEVELS OBSERVED IN YOUNG MALE RHESUS MACAQUES

Supplementation with DHEA resulted in a significant increase in circulating DHEAS in old males shortly after administration (**Figure 2A**, closed circles). Interestingly, although DHEAS levels declined throughout the day, they again began to increase shortly after administration of T at 1900 h. This increase was sustained throughout the night, resulting in higher DHEAS levels than baseline in the morning before DHEAS had been administered.

The supplementation paradigm was modified to further explore this phenomenon. If DHEAS rose throughout the night in the absence of exogenous T, we could conclude that this is a possible priming effect, by which exogenous DHEA stimulates the adrenal glands to produce more of their own DHEAS for the following day. However, as shown in **Figure 2B**, circulating DHEAS remained low throughout the night when no T was administered. A repeated-measures ANOVA with Greenhouse-Geisser correction for differences in sphericity was performed, comparing DHEAS between 1900 and 0700 h in animals receiving both T and DHEA as described versus animals receiving DHEA in the morning but no T at 1900 h. This test revealed a significant effect of time ($F = 4.978$, $P = 0.026$), treatment ($F = 20.786$, $P = 0.003$), and treatment-by-time interaction ($F = 10.640$, $P = 0.002$).

DHT ADMINISTRATION IN AGED MALE RHESUS MACAQUES SIGNIFICANTLY INCREASES CIRCULATING DHEAS TO LEVELS SEEN IN YOUNG MALE RHESUS MACAQUES

The results from the previous experiments suggest that T itself can increase circulating DHEAS. Testosterone is only two steps beyond DHEA in the steroidogenic pathway, and our dose of T needed to be very high in order to increase circulating levels to those of young animals. Therefore, we hypothesized that some of the exogenous T may have been back-converted to DHEAS, resulting in the observed gradual increase in circulating DHEAS levels. To test this possibility we replaced the exogenous T in our androgen supplementation paradigm with its more active metabolite, DHT. We reasoned that if our administered T was being converted back to DHEAS, then substitution of T with DHT, a hormone further along the steroidogenic pathway, should prevent the increase in DHEAS levels from occurring. However, the results clearly show that even with this alternate androgen supplementation paradigm circulating DHEAS increased above baseline levels starting at 1900 h; the resulting hormone pattern was nearly identical to that of young control animals (**Figure 2C**). A repeated-measures ANOVA between the baseline and DHT-supplemented animals revealed a significant effect of group ($F = 12.944$, $P = 0.009$), with DHT treatment resulting in an increase in circulating DHT starting at 1900 h. These data suggest that not only is a back-conversion

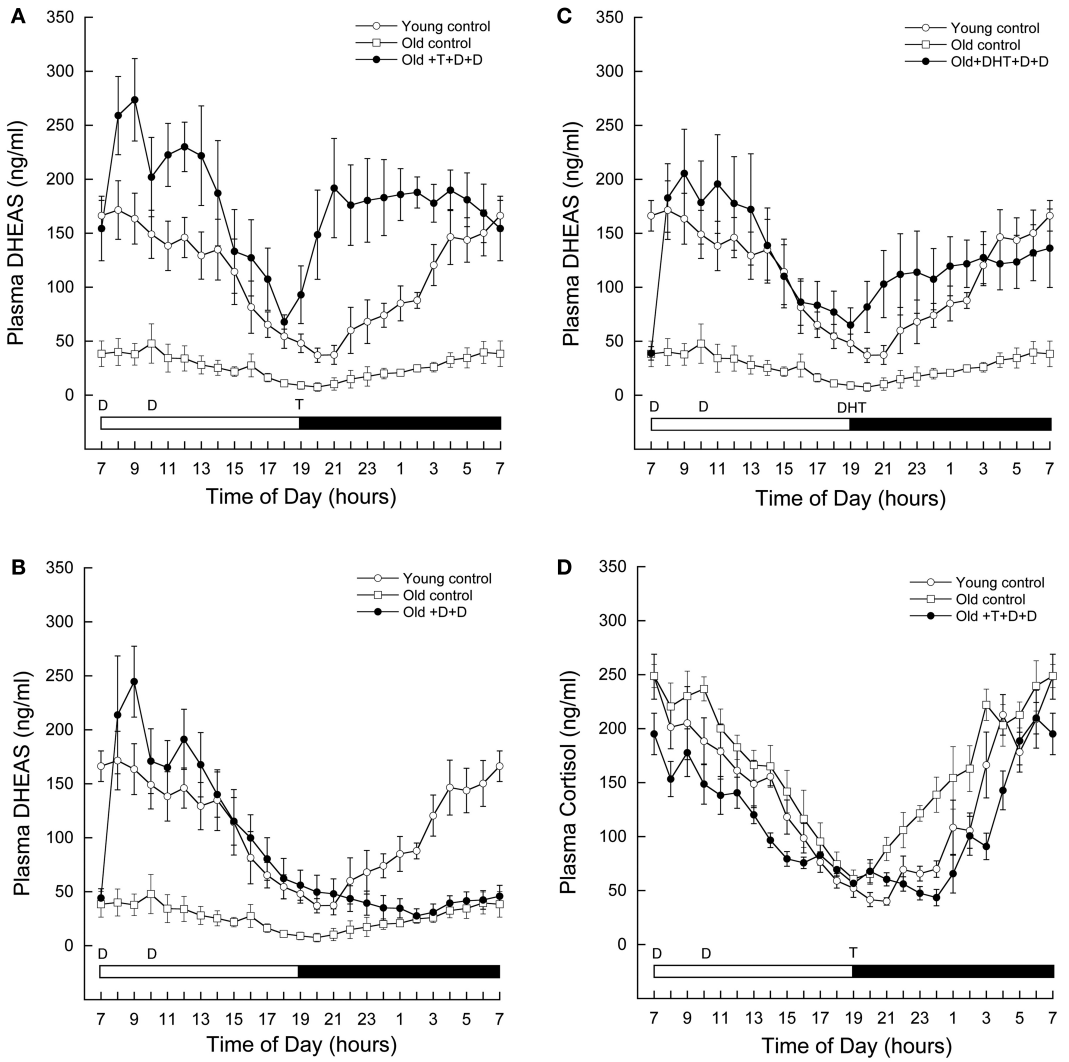

FIGURE 2 | Age-related changes in circulating adrenal steroid levels in male rhesus macaques, and the impact of androgen supplementation. Serial blood samples were remotely collected from young (7–12 years, $n = 4$) and old (21–26 years, $n = 4$) males, as well as old males exposed to 5 days of various androgen supplementation paradigms (21–26 years, $n = 5$). The samples were subsequently assayed for DHEAS **(A–C)** and/or cortisol **(D)**. Each data point represents the mean and vertical lines indicate the SEM. Time of day is indicated along the abscissa, and periods of light and darkness are represented by white and black bars, respectively. In each panel, the times of oral androgen administration are depicted as follows: D = DHEA (0.04–0.10 mg/kg body weight), T = testosterone (12 mg/kg body weight), DHT = 5α-DHT (5–10 mg/kg body weight). For reference, the same DHEAS profiles from the young and old controls are depicted in **(B–C)**. The data demonstrate that androgen supplementation at an appropriate time of day can restore 24-h circulating DHEAS levels in old males rhesus macaques. Importantly, they also demonstrate an unexpected stimulatory action of gonadal steroids on DHEAS.

of T to DHEA/S unlikely, but that the increase in circulating DHEAS is an androgen-receptor-mediated mechanism.

TESTOSTERONE AND DHEA SUPPLEMENTATION DO NOT CAUSE AN INCREASE CIRCULATING CORTISOL

Both DHEA and cortisol are secreted by the adrenal gland in response to adrenocorticotropic hormone (ACTH) from the hypothalamus. To further examine where testosterone is having an effect to increase DHEAS, we also assayed a series of 24 h blood samples from our DHEA and T combined supplementation paradigm for cortisol. If androgen activation at the level of the brain increases ACTH secretion, cortisol would also increase at the time of T supplementation. However, as shown in **Figure 2D**, T supplementation did not result in increased circulating cortisol levels. A repeated-measures ANOVA with time as a within-subjects factor and group (old control, open circles; old androgen-treated, closed circles; and young, open squares) as a between subjects factor revealed a significant effect of time ($F = 56.758$, $P < 0.001$), group ($F = 5.438$, $P = 0.028$), and group-by-time interaction ($F = 2.093$, $P < 0.001$). The significant effect of group was driven by differences between the old control and old androgen-treated animals, with androgen treatment associated with significant reductions

in circulating cortisol at 1000, 1500, 2100, 2200, 2300, 2400, and 0300 h. There was no significant difference between circulating cortisol in the young animals as compared to either the old control or old androgen-treated animals.

DISCUSSION

Research on adrenal sex differences in humans and non-human primates is limited, but some interesting observations have been made. The finding that DHEA/S differs dramatically between males and females is highly consistent and is maintained throughout the lifespan in both humans and rhesus macaques (7, 20–22), but to date no theories as to the mechanism of this difference have been adequately investigated. The differences in aging profiles of adrenal and gonadal hormones further complicates any potential interactions, as estrogen in females drops precipitously at the time of menopause, DHEA/S in both sexes declines slowly and consistently starting in the third decade (8), and testosterone in males decreases very slowly, gradually, and to a much lesser extent than other hormones (23–25).

While much work is yet to be done to study differences between the male and female adrenal gland, early work suggests that the difference in adrenal output is not due to intrinsic physiological differences but due to differential hormonal input to the adrenal glands of males and females. Although sex differences in both circulating DHEA/S and adrenal morphology and physiology are seen in the marmoset, with respect to both size of the zona reticularis (ZR, the layer of the adrenal gland that synthesizes DHEA/S) and expression of steroidogenic genes, similar differences are not observed in humans. Specifically, in the marmoset, females secrete significantly more DHEA/S than males due to both gross anatomical differences in the adrenal gland with increased adrenal zonation, as well as differences in enzymatic machinery with an increased expression of cytochrome b5 (26). However, in humans the male and female ZR are similar in both size and cytochrome b5 expression (27). Also, cultured male and female adrenal glands respond with identical levels of DHEA secretion when stimulated by ACTH (2). Further, females receiving long-term treatment with T show an increased response of DHEA to ACTH stimulation as compared to female controls (28, 29), suggesting that the adrenal machinery is the same in males and females, but the hormonal input from the HPG axis can modulate adrenal output. Given the established research on male and female adrenal physiology and the lack of evidence for sex differences in either gross morphology or cellular physiology, it is our hypothesis that the higher level of DHEAS seen in male humans and non-human primates is due to T increasing the sensitivity of the ZR to ACTH. Consistent with this hypothesis, our paradigm of testosterone supplementation significantly increased circulating DHEAS of aged male macaques in a manner mimicking the circadian profile of DHEAS in young male macaques.

Although we cannot rule out the possibility that our androgen supplementation paradigms acted further up in the hypothalamo-pituitary–adrenal axis, it is unlikely that CRH or ACTH were affected because we saw no stimulatory effect of androgen on cortisol; like DHEA/S, cortisol is stimulated by ACTH but is secreted primarily from the zona fasicularis (ZF) rather than the ZR. Also, several studies suggest ACTH secretion is similar in males and females (30–33). Previous studies of post-menopausal women found that long-term administration of DHEA increased DHEA production in response to ACTH (34, 35), a finding we did not replicate presently as when DHEA was administered without testosterone we observed no night-time increase in circulating DHEAS. However, our study supplemented animals for less than 1 week, which may not have been enough time to adequately increase adrenal sensitivity to ACTH. Thus, it is likely that increased levels of both DHEA/S and T can induce the adrenal glands to produce more DHEA/S.

One endogenous disturbance of normal androgen interactions can be seen in the case of polycystic ovarian syndrome (PCOS), in which women exhibit high levels of both testosterone and DHEA/S, and these women demonstrate an increased responsiveness of DHEAS production when stimulated with ACTH (36). High levels of testosterone have in fact been implicated as a potential cause of PCOS (37, 38); however, the impact of increased testosterone on circulating DHEA/S in women has yet to be studied extensively.

One of the most common complaints in aging is a decline in cognition (39, 40), a domain that has been studied extensively with regard to estrogens, T, and DHEA/S. While some success has been seen with estrogen replacement in younger women post-ovohysterectomy (41), and with testosterone supplementation in elderly men (42–44), results from large-scale HT studies are bleak at best (45, 46). Despite promising results in rodents and benefits of the HT on other target tissues, such as maintenance of muscle mass (47), bone density (48, 49), and immune function (50, 51), DHEA/S supplementation in elderly humans (52–56) shows little to no effect on cognition. A potential drawback of all of these studies is their focus on just one component of the endocrine system. As these hormones interact, changing one steroid may result in compensation by others, rendering the effects null. Therefore, a combination of adrenal and gonadal HT may be more promising for the cognitive domain than HT with any one steroid alone.

Our results indicate that not only does the interaction between DHEAS and T occur in the adrenal gland (and not the hypothalamus, as higher ACTH would increase circulating cortisol), but it is limited to an effect on the ZR, the area that produces DHEA/S, and not the ZF, the area that synthesizes cortisol, as cortisol levels were not affected by T administration. Additionally, DHEAS, but not cortisol, decreased with age, suggesting changes in only the ZR occur with age. This is consistent with studies showing regression of the ZR in older humans (27, 57) and rhesus macaques (5). However, the ability of the adrenal gland to respond to T administration with youthful production of DHEAS suggests DHEA/S production itself is not impaired with age, but the responsiveness of the ZR to ACTH may be dampened. Thus, it may not be necessary to supplement with DHEA itself to increase circulating DHEAS levels in the elderly; merely supplementing with T with the physiologically correct time course may help to restore an overall youthful hormonal profile in elderly men.

AUTHOR CONTRIBUTIONS

Krystina G. Sorwell contributed to the study design, data collection, data analysis, interpretation of results, and writing and revising the manuscript. Dr. Steven G. Kohama contributed to

the study design, interpretation of results, and assisted in revising the manuscript. Dr. Henryk F. Urbanski contributed to the study design, data collection, interpretation of results, and assisted in writing and revising the manuscript.

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Gender effects and sexual-orientation impact on androstadienone-evoked behavior and neural processing

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In humans, the most established and investigated substance acting as a chemosignal, i.e., a substance that is excreted from the body, is 4,16-androstadien-3-one (AND). AND, which is found in sweat and saliva, is known to be responsible for influencing several variables, such as psychophysiological status, behavior, as well as cortical processing. The aim of the present review is to give insight into the variety of AND effects, with special regard to specific cross-sexual characteristics of this putative human chemosignal, emphasizing the neural activation patterns and factors such as contextual conditions. This review highlights the importance of including those contributing factors into the analysis of behavioral as well as brain-related studies.

Keywords: androstadienone, chemosignals, gender-effect, sexual-orientation, neuronal processing, psychophysiological status, behavior

INTRODUCTION

There is evidence for a sexual dimorphism of the brain (e.g., Coffey et al., 1998; Sacher et al., 2013) and new medical theories point out the importance of gender specific medicine in terms of risk-factors, epidemiology and treatment outcomes (e.g., Mielke et al., 2014). In order to extract essential and comprehensive information from behavioral as well as functional imaging studies, including gender, as an essential factor is of great interest (Sacher et al., 2013). This review highlights this importance for chemosensory science in which chemosignals are known to evoke a gender specific effect themselves.

The term "pheromones" (so-called chemosignals) was coined in 1959 by Peter Karlson and Martin Lüscher, who defined them as "substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example a definite behavior or a developmental process" (Karlson and Lüscher, 1959). The search for these semiochemicals is still an elusive goal of chemical ecology and communication studies. Today, it is clear that chemosignals play a significant role in social interaction and communication in various species. It is well known that animals are able to communicate affective states, such as stress, alarm, fear, anxiety, or sexual interest, by manipulating chemosignals produced from their skin (Kiyokawa, 2004; Kiyokawa et al., 2006). In animals, these signals are jointly processed by the vomeronasal organ (VNO) and by the accessory olfactory systems, as well as by the main olfactory system (Chamero et al., 2012; Petrulis, 2013). The wide range of literature addressing the VNO provides little consensus about the presence of the VNO in humans (for a review, see Meredith, 2001). While a structure similar to the VNO of animals was found *in utero* in humans (Knecht et al., 2003), the same structure is not continuously noticeable in adults (Trotier et al., 2000; Trotier, 2011). To date, it is not clear whether a human VNO exists and whether it plays a role in the perception of chemosignals (Frasnelli et al., 2011).

Several areas of the human body—such as feet, mouth, or, particularly, axillary regions—are known to produce odors that act as chemosignals (Pause, 2012). Recently, Gelstein et al. (2011) found that tears convey chemosignals, and even ear wax was proposed to be able to transport chemosensory information (Prokop-Prigge et al., 2014). Nevertheless, human sweat is the most extensively investigated conductor of chemosignals (Porter and Moore, 1981; Lundström et al., 2008; Zhou and Chen, 2008; Zernecke et al., 2010; Albrecht et al., 2011).

Although the human body odor cocktail can contain well over 200 individual components (Zeng et al., 1996), the most intensively studied component is the steroid 4,16-androstadien-3-one (AND). The chemical structure of the molecule AND is very similar to androstenon, a well-known animal pheromone (Melrose et al., 1971). AND's specific cross-sexual characteristics and its impact on human behavior and psychophysiological events, in particular, have drawn much attention in recent research.

The following sections will focus on research addressing the behavioral and psychophysiological effects as well as the neuronal processing of AND, emphasizing its gender-specific and cross-sexual characteristics.

BEHAVIORAL AND PSYCHOPHYSIOLOGICAL EFFECTS OF *AND*

AND is one of the substances known to modulate psychological and physiological states, as well as human behavior in a non-conscious manner (Lundström and Olsson, 2005). It is known that AND is associated with specific cross-sexual characteristics and has recently attracted much attention, particularly regarding its effect on women's psychophysiology (Jacob and McClintock, 2000; Jacob et al., 2001, 2002; Lundström and Olsson, 2005; Wyart et al., 2007). However, there is evidence that AND influences men's psychophysiology and behavior as well (Bensafi et al., 2003, 2004). These gender-specfic effects not only make AND particularly interesting, but also induce an interpretation bias of certain results due to mixed study groups and inextensive study description. **Table 1** gives an overview of gender-specific studies and findings.

The data from previous behavioral and psychophysiological studies are provided in the following sections categorized according to the gender of the sample.

STUDIES INVESTIGATING *AND* IN A FEMALE SAMPLE

Psychophysiological manifestations related to AND exposure in female subjects have been reported, based on AND detected in salivary cortisol levels (Wyart et al., 2007) and autonomic physiology levels showed a significant reduction in respiratory and cardiac frequency, as well as skin conductance and increased body temperature (Grosser et al., 2000). Beyond that, Lundström and colleagues suggested that AND enhanced women's feeling of being focused (Lundström et al., 2003) and induced an increased attentiveness, even outside the conscious detection of AND (Lundström and Olsson, 2005). In addition, analyses of chemosensory event-related potential (ERP) recordings revealed AND to be processed between 13 and 20 percent faster than odorants similar in hedonic and intensity ratings (Lundström et al., 2006b). With special regard to AND's generally proposed role in reproductive behavior, two studies (Thorne et al., 2002; Saxton et al., 2008) indicate that men's ratings of female attractiveness are modulated by this special chemosignal with higher evaluations of women in the AND condition, while another experiment (Lundström and Olsson, 2005) was not able to confirm this effect.

Saxton et al. (2008), who assessed the effect of AND in a speed-dating-event, argued that the suggested context-dependency of AND may occur only in the presence of a male person (Jacob et al., 2001; Lundström and Olsson, 2005). Furthermore, recent literature suggests that AND strenghtens intrasexual competition strategies in women (Parma et al., 2012).

The role of AND as modulator-chemosignal has also been discussed in the context of serving as a link between hormonal status and this special steroid: While women taking oral contraceptives are more sensitive to environmental odors, fertile women showed a higher sensitivity to chemosignals with reproductive relevance, like androstadienone (Lundström et al., 2006a). With regard to AND's influence on mood, AND was found to enhance a positive mood in women, with feelings of being more relaxed, calm, and free of negative feelings (Grosser et al., 2000; Preti et al., 2003). Another study reported that the setting, the manner, and by whom the experiment was conducted play a role in perception. Lundström and Olsson (2005) emphasized the impact of socioexperimental conditions in women who showed changes in self- reported mood only when experimental interactions were completed by a male experimenter, thus, again, proposing a context-dependent effect for AND.

STUDIES INVESTIGATING *AND* IN A MALE SAMPLE

Studies using male samples are rare. The only study investigating the effect of AND in men came out recently, and demonstrated that AND directly affected mens' cooperative behavior by increasing such behavior (Huoviala and Rantala, 2013).

STUDIES INVESTIGATING *AND* IN MIXED SAMPLES

We defined studies with mixed samples as those in which the study design included men and women.

With regard to the activational effects in the sympathetic nervous system, a study by Jacob et al. (2001) was able to confirm the suggested calming effects of AND on women's physiology, as already suggested by a study using the female sample presented in the above section (see Section "Studies Investigating AND in a Female Sample"; Grosser et al., 2000). While AND administration led to raised skin temparature in men and lowered temperature in women, AND increased skin conductance in both sexes, with a significantly higher effect observed in women, indicating that the arousing effect was more prevalent in women than in men (Jacob et al., 2001). Interestingly, the activational effects of AND were dependent on the socioexperimental context, since women's reactions were observed only in sessions administered by a male investigator. Some other findings emphasized the context-dependency of AND in a similar fashion. While AND administered in a neutral context, or in a context with little social interaction, did not influence autonomic nervous system functions, it enabled increased sexual arousal in a sexually arousing context in a sex-independent manner (Bensafi et al., 2004; Hummer and McClintock, 2009). During the same sexually arousing context, respiration rates, especially in men, decreased, while skin temperature in both sexes rose (Bensafi et al., 2004).

Concerning psychological variables, AND was reported to have divergent effects in men and women, as shown for psychophysiological states; while AND administration led to increased negative emotions in men (Jacob and McClintock, 2000), especially in unpleasant settings (Bensafi et al., 2004), no negative effect was evoked in women. Focusing on the context-dependency of AND, positive feelings in women were found to be sustained during a sad time (Bensafi et al., 2004) and were increased in a neutral context (Jacob and McClintock, 2000).

Further, another study that analyzed the effect of AND on mood and pain perception concluded that exposure to this steroid led to an amelioration of mood state only in women (Villemure and Bushnell, 2007). Based on this finding, the authors further hypothesized that women would show lower pain sensation when exposed to AND. However, this assumption was not confirmed, as women, interestingly, showed increased percieved pain (Villemure and Bushnell, 2007).

An effect of the experimenters sex on the baseline response in an AND experiment has recently also been observed

Table 1 | Behavioral and psychophysiological results induced by *AND*.

		References
RESULTS OF STUDIES INVESTIGATING *AND* IN A FEMALE SAMPLE		
– Reduction of respiratory and cardiac frequency as well as skin conductance and increased body temperature		Grosser et al., 2000; Lundström and Olsson, 2005
– Higher salivary cortisol levels		Wyart et al., 2007
– Increases feeling of being focused		Lundström et al., 2003 Lundström and Olsson, 2005
– More intense pain perception		Villemure and Bushnell, 2007
– Intensifies intrasexual competition strategies		Parma et al., 2012
– Higher attractiveness ratings of men		Saxton et al., 2008
– Enhances positive mood (feelings of being more relaxed, calm, and free of negative feelings)		Grosser et al., 2000 Lundström and Olsson, 2005
– Higher sensitivity to AND in fertile women		Lundström et al., 2006a
– Induces faster and more pronounced cortical responses		Lundström et al., 2006b
RESULTS OF STUDIES INVESTIGATING *AND* IN A MALE SAMPLE		
– Increases cooperative behavior		Huoviala and Rantala, 2013
RESULTS OF STUDIES INVESTIGATING *AND* IN MIXED SAMPLES		
Women	**Men**	
– Increases sexual arousal and skin temperature in a sexually arousing context	– Increases sexual arousal and skin temperature in a sexually arousing context – Decreases respiration rates in a sexual arousing context	Bensafi et al., 2004
– Decreases skin temperature and increases skin conductance	– Increases skin temperature and skin conductance	Jacob et al., 2001
– Increased positive feelings	– Increased negative feelings (especially in unpleasant settings)	Jacob and McClintock, 2000; Bensafi et al., 2004
– Increased pain perception		Villemure and Bushnell, 2007

in rodents (Sorge et al., 2014). Regarding future research, preliminary findings about the sex-divergent effects of AND on psychophysiological as well as psychological variables should be considered, especially the context-dependency of AND. This context-dependency should implicitly be noted when planning experiments, as well as interpreting results to prevent an interpretation bias.

NEURONAL CORRELATES OF *AND*

In the past few years, a great number of neuroimaging studies have provided insight into the neuronal processing of common odorants by the olfactory pathway (for a review, see Lundström et al., 2011). Whereas common odors normally activate the temporal-frontal junction, the so-called piriform cortex, amygdala, insula, and the orbitofrontal cortex, body odors are commonly found to trigger a network located outside the main olfactory system, including the posterior cingulate cortex, the occipital gyrus, the angular gyrus, and the anterior cingulate cortex (for a review, see Lundström and Olsson, 2010). The following section aims to provide the reader with an overview about the neuronal processing of the chemosignal AND.

Neuroimaging studies have illustrated a gender-specific outcome, but another significant finding should be specifically noted—the sexual-orientation effect. As a result of this, the following sections will be segmented into gender-specific neuronal processing and the impact of sexual orientation.

To illustrate the findings discussed in the subsequent paragraphs more clearly, we provide the reader with an overview of neural activation patterns induced by AND, classified by gender and sexual orientation (see **Figure 1**).

GENDER-SPECIFIC NEURONAL PROCESSING OF *AND*

Early positron emisson tomography (PET) studies exploring the neural correlates of AND perception suggested the presence of gender-specific neural activation in which the hypothalamic pathway was significantly activated in heterosexual women, but heterosexual men lacked this hypothalamic activation, and areas of the olfactory cortex were activated instead (Savic et al., 2001, 2005, 2009; Berglund et al., 2006, 2008; Hillert et al., 2007; Ciumas et al., 2009). However, contrary to these PET studies, a recent fMRI study found non-sex-specific hypothalamic activation (Burke et al., 2012). When the authors applied various concentrations of AND, significantly higher hypothalamic activation was demonstrated in women than in men when a higher concentration was used (10 mM). This corresponds with previous studies. However, when participants were exposed to

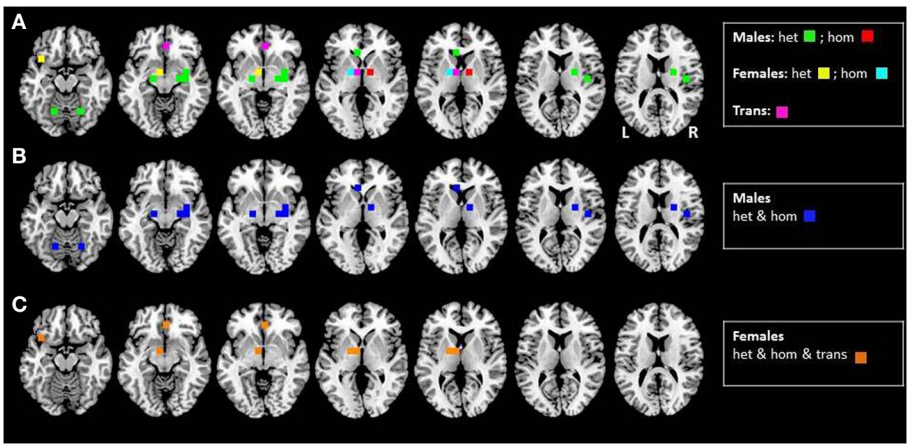

FIGURE 1 | Axial mean anatomical images overlaid with neural activation induced by AND, resulting from different functional imaging studies (see Table 2). In order to enhance comparability, we included all positron emission tomography (PET) studies using the same tracer, with the special pheromone-like compound that induced neuronal activation (see **Table 2**). Since results from certain subject groups were re-utilized as controls throughout these studies, these activations are illustrated once. Voxels were highlighted with a 10 mm sphere. To illustrate the impact of sexual orientation, activations in heterosexual (green) and homosexual (red) men, in heterosexual (yellow) and homosexual (cyan) women, as well as in non-homosexual male-to-female transsexuals, are mapped separately **(A)**. Sex-specific differences in activation patterns are shown in **(B)** (hetero- and homosexual males; blue) and **(C)** (hetero- and homosexual females, as well as male-to-female transsexuals; orange).

Table 2 | Overview of studies included in graphic design.

References	N (total)	Healthy adults		Modality	Stereotactic space	Contrast	Tracer
		Age (y; range)	Subgroups (sexual orientation)				
Berglund et al., 2006	36	33 ± 6	12 HeM	PET	TAL	AND > AIR	^{15}O-H_2O
		28 ± 2	12 HoW				
		26 ± 2	12 HeM				
Berglund et al., 2008	36	33 ± 6	12 HeW	PET	TAL	AND > AIR	^{15}O-H_2O
		26 ± 2	12 HeM				
		32 ± 8	12 MFTR's				
Ciumas et al., 2009	26	26 ± 7; 20–36	13 HeW	PET	TAL	AND > AIR	^{15}O-H_2O
		28 ± 6; 21–36	13 HeM				
Hillert et al., 2007	12	26 ± 3; 20–28	12 HeW	PET	TAL	AND > AIR	^{15}O-H_2O
Savic et al., 2001	24	20–28	12 HeW	PET	TAL	AND > AIR	^{15}O-H_2O
		23–28	12 HeM				
Savic et al., 2005	36	26 ± 2	12 HeW	PET	TAL	AND > AIR	^{15}O-H_2O
		28 ± 2	12 HeM				
		33 ± 7	12 HoM				
Savic et al., 2009	12	21-36	12 HeM	PET	TAL	AND > AIR	^{15}O-H_2O

HeW, Heterosexual women; HeM, Heterosexual men; HoW, Homosexual women; HoM, Homosexual men; MFTR's, Non-homosexual male-to-female transsexuals.

medium concentrations (0.1 mM), men demonstrated a significantly stronger hypothalamic response than the participating women (Burke et al., 2012). These results led the authors to conclude that AND evokes hypothalamic responses in both sexes in a stimulus concentration-dependent manner. However, when comparing these results to the series of studies by Savic and coworkers (Savic et al., 2001, 2005, 2009; Berglund et al., 2006, 2008; Hillert et al., 2007; Ciumas et al., 2009), it should be noted that Burke et al. (2012) used other odor delivery methods, compound concentrations, as well as another imaging technique, all of which together affected our ability to directly compare results.

IMPACT OF SEXUAL-ORIENTATION ON NEURONAL PROCESSING OF *AND*

The last decade of brain imaging research has revealed that AND stimulation produces significant and localized group effects that are seemingly sexual orientation-dependent. There are several functional neuroimaging studies, which address cortical

responses to pheromones that seem to be dependent on sexual orientation; homosexual men and male-to-female transsexuals were found to display the same activation pathway as heterosexual women, i.e., demonstrating hypothalamic responses upon exposure to AND (Savic et al., 2005; Berglund et al., 2008).

In contrast, Berglund and colleagues concluded that homosexual women process AND similar to heterosexual men, namely, by parts of the olfactory cortex (Berglund et al., 2006). Those findings (Savic et al., 2005; Berglund et al., 2006, 2008), are of great importance in order to underline existing evidence of sexual-orientation effects on neural processing in other research areas as well. Recently, behavioral and neuroimaging results from Perry et al. (2013) showed empathy to be related to gender, as well as sexual preference. Further, a study aiming to characterize regional homogeneity and functional connectivity during rest found significant differences between homo- and heterosexual men (Hu et al., 2013). Hence, it would be presumptuous not to consider the ability to use this variable as a modulating factor to achieve homogeneous subject groups.

AND-EST INCONSISTENCY

As noted above, AND is processed in a gender-specific manner; however, thus far, few studies have dealt with the functional aspects beyond neural activity. Most published studies investigating the effect of AND on neural processes also included another potential human pheromone, namely estra-1,3,5(10),16-tetraen-3-ol (EST) (Savic et al., 2001, 2005, 2009; Berglund et al., 2006, 2008; Hillert et al., 2007; Ciumas et al., 2009). EST has, among others, been detected as a natural component of the urine in pregnant women (Thysen et al., 1968). Interestingly, the administration of this estrogen-like steroid causes an effect complementary to AND. Whereas AND application caused a hypothalamic activation in women and the activation of common olfactory areas in men, EST is processed in a diametrically opposite fashion, namely, via the hypothalamic pathway in heterosexual men and parts of the olfactory cortex in heterosexual women (Savic et al., 2001, 2005, 2009; Berglund et al., 2006, 2008; Hillert et al., 2007; Ciumas et al., 2009). This complementary relationship seems to be of great physiological relevance.

Compared to AND, specific psychophysiological measures of EST are largely unknown. However, while the results of Bensafi et al. (2003) revealed no effect of EST on physiological arousal, the same authors found EST to affect physiological arousal in a content-dependent way (Bensafi et al., 2004). Exploring the conscious odor perception of AND and EST in an animal model, a gender-specific effect was obtained by Laska et al. (2006), who detected olfactory sensitivity to AND in female, but not male, spider monkeys, while responses to the highest concentrations of EST were found in males, but not in female, monkeys. These data also highlight the gender-specific processing of these two pheromone-like compounds in non-human animal models. Finally, these results indicate that there is a considerable need for research on the psychophysiological effects of EST.

FINAL REMARKS

As shown, non-conscious application of AND mediates human behavior, psychophysiology, as well as cortical processing, with different responses and activations in men and women. With regard to previous findings, the suggested influence of contextual condition should be considered in any further planning of a trial, as well as in the interpretation and reporting of results. In addition, the discrepancies in results between various studies further emphasize the need for further research in this area, especially in the field of sub- and suprathreshold application of AND to rule out potential concentration-dependent effects. Moreover, the advent of chemosensory imaging using fMRI allows a more stringent and temporally detailed investigation of the neural processing of AND. Finally, as demonstrated by the neuroimaging results presented above, linked to sexual orientation, care should be taken to either include homogeneous subject groups or to carefully control for demographic characteristics. The mediating mechanisms of these sex and sexual preference-specific effects on behavioral, psychophysiological, and neural processing of AND should form the basis of further research.

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Sex-specific strategy use and global-local processing: a perspective toward integrating sex differences in cognition

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This article reviews the literature on sex-specific strategy use in cognitive tasks with the aim to carve out a link between sex differences in different cognitive tasks. I conclude that male strategies are commonly holistic and oriented toward global stimulus aspects, while female strategies are commonly decomposed and oriented toward local stimulus aspects. Thus, the strategies observed in different tasks, may depend on sex differences in attentional focus and hence sex differences in global-local processing. I hypothesize that strategy use may be sex hormone dependent and hence subject to change over the menstrual cycle as evidenced by findings in global-local processing and emotional memory. Furthermore, I propose sex hormonal modulation of hemispheric asymmetries as one possible neural substrate for this theory, thereby building on older theories, emphasizing the importance of sex differences in brain lateralization. The ideas described in the current article represent a perspective toward a unifying approach to the study of sex differences in cognition and their neural correlates.

Keywords: sex differences, cognitive functions, Hemispheric asymmetries, sex hormones, cognitive strategies, global-local processing

HE OR SHE—SHORT INTRODUCTION TO SEX DIFFERENCES IN COGNITION

For decades it has been debated, whether men and women differ in specific cognitive abilities (e.g., Halpern, 2000; Hyde, 2005; Andreano and Cahill, 2009). The present chapter shall by no means provide an extensive review of these sex differences, but rather introduce those sex differences, which are relevant for the idea presented in this article.

Sex-sensitive tasks have been identified in the domains of spatial, verbal, and memory performance. Thereby the largest sex differences, indicating a male superiority, have been reported for mental rotation tasks (e.g., Voyer and Bryden, 1990; Voyer et al., 1995; Schoning et al., 2007) with effect sizes ranging from 0.25 to 3.04 (Andreano and Cahill, 2009) and spatial navigation tasks (e.g., Galea and Kimura, 1993; Silverman et al., 2000; Saucier et al., 2002) with effect sizes ranging from 0.36 to 1.04 (Andreano and Cahill, 2009). In the domain of verbal abilities, sex differences have been reported for verbal fluency tasks (e.g., Capitani et al., 1998, 1999, 2005) with effect sizes ranging from 0.13 to 0.89 (Mann et al., 1990; Bolla et al., 1998; Loonstra and Sellers, 1998; Halari et al., 2005, 2006; De Frias et al., 2006; Gauthier et al., 2009; Hausmann et al., 2009; Soleman et al., 2013; Hirnstein et al., 2014), verbal memory tasks (e.g., Kimura and Seal, 2003; Yonker et al., 2003) with effect sizes ranging from 0.18 to 0.97 (Andreano and Cahill, 2009). But non-significant effects and effects to the opposite have also been reported (e.g., Kimura and Seal, 2003; Yonker et al., 2003; Munro et al., 2012). Sex differences have also been observed in autobiographic memory (Pillemer et al., 2003) and other aspects of episodic memory, like the recognition of odors (e.g., Oberg et al., 2002), faces (e.g., Bengner et al., 2006), objects, and pictures (e.g., Galea and Kimura, 1993), as well as for a variety of higher order cognitive functions (Harness et al., 2008; Li et al., 2009; Huster et al., 2011).

However, several of these findings have been questioned by theorists arguing that sex differences are overall small and negligible and within-group variation is stronger than the variation between groups (Hyde, 2006; Hyde and Linn, 2006). For example, a meta-analysis by Hyde and Linn (1988) yielded only a very weak effect size ($d = 0.11$) for sex differences in verbal functions across 165 studies using different verbal tasks. However, whether overall performance differences between men and women exist, does not explain why and how cognition differs between the sexes.

First, there is an increasing interest in the actions of sex hormones on the brain (De Frias et al., 2006), which may affect sex differences in cognitive tasks, emotional processing or personality. However, sex hormone levels, especially in women are not constant, but subject to changes due to endogenous hormonal fluctuations (menstrual cycle) or the application of synthetic steroids (hormonal contraception). Hence, for some tasks we do of course expect within-group variation in cognitive performance that may mask differences between groups, if these factors are not adequately controlled for (see also Pletzer et al., 2011, 2014a). It has for example been demonstrated that women perform better on mental rotation and other spatial tasks during the early follicular phase (low estrogen and progesterone) or if on hormonal contraception, while verbal abilities are also increased in hormonal contraceptive users as well as during the luteal cycle phase (high estrogen and progesterone) (Hampson, 1990;

e.g., Mordecai et al., 2008; Wharton et al., 2008; Dadin et al., 2009). However, as outlined by a recent review (Sundstrom et al., this topic), these findings are not always replicable, also due to inconsistencies in the definition of cycle phases.

Second, it has been argued in the personality domain, that one cannot conclude from the comparison of isolated personality dimensions that differences between men and women in personality are small (Del Giudice et al., 2012). Rather personality has always been viewed as a multidimensional construct and research on sex differences should approach it as such. Personality dimensions do not affect our behavior individually. It is their relative manifestation and the interactions between different traits that form our behavior as a whole and these relations and interactions are what should be studied with respect to individual differences, as e.g., sex differences. I propose that the same holds true for the cognitive domain. While sex differences in some abilities may be small and sex differences are more pronounced in some abilities than others (e.g., spatial abilities), it is our cognitive profile, i.e., the common action and interaction of all aspects of cognition that shape our everyday life performance. Therefore, research on sex differences should advance from studying some cognitive abilities as separate entities, but move toward a more integrative approach and try to link sex differences across singular tasks. By identifying similarities between sex differences in various tasks, we may derive common principles and ideally link these principles to neural substrates.

Third, an absence of sex differences at the behavioral level does not necessarily imply that men and women did process a specific task in the same way. For example, several neuroimaging studies demonstrate sex differences in brain activation during a task, while not observing behavioral sex differences (e.g., Weiss et al., 2003; Schoning et al., 2007). Many authors have argued that men and women use different cognitive strategies (e.g., Cochran and Wheatley, 1989) employing different approaches. A common method is to utilize different instructions or the use of different stimulus materials or categories that favor the use of one strategy over another. For example, Sharps et al. (1993) were able to demonstrate that sex differences in a mental rotation task disappear, if they used non-spatial instructions and a female superiority in object location memory disappears, if the labeling of items is not possible (Postma et al., 2004). The use of different strategies may also be suggested by eye-tracking studies, if men and women focus on different stimulus aspects (e.g., Hampson, 1990). Another source of information regarding sex-specific strategy use are participants self-reports (e.g., Gluck, 2003). The following chapter will summarize several examples, where men and women approach a task with different strategies. As I will outline, it may be the nature of these differential strategies that links sex differences across tasks and to brain organization.

HOLISTIC OR DECOMPOSED—DISSOCIATION OF SEX-SPECIFIC STRATEGIES ACROSS TASKS

The dissociation of cognitive strategies between men and women has been studied most extensively in spatial tasks. In the mental rotation task for example, it has been deduced from participants self-reports that men tend to use a more holistic "Gestalt" approach, while women use a segmentary strategy of rotating parts of the stimuli separately (Gluck, 2003; Pena et al., 2008; Rilea, 2008). In spatial navigation, it has been demonstrated using participants self-reports and different instructions, that men focus on distal landmarks and use allocentric coordinates, while women focus on local landmarks and use egocentric coordinates (Galea and Kimura, 1993; Lawton, 1994, 2001; Lawton et al., 1996). Men outperform women in both real world and 2D-matrix navigation, when directions are given in Euclidean terms using allocentric coordinates (Saucier et al., 2002). On the other hand, women outperform men, when directions are given using landmark information and egocentric coordinates (Saucier et al., 2002) and sex differences in virtual navigation decline the more landmark information is available (Andersen et al., 2012). For navigation, the sex-specific strategy dissociation has been corroborated by eye-tracking evidence. In a virtual water maze, men explore more space, while women show longer fixation durations (Mueller et al., 2008). Furthermore, the allocentric strategy has successfully been related to mental rotation performance (Saucier et al., 2002), demonstrating that a more global strategy is beneficial in spatial tasks.

A dissociation between global and local strategies has however also been described for a variety of spatial-related and non-spatial tasks. For example, using different stimulus categories, we recently described a strategy dissociation in a number comparison task (Pletzer et al., 2013), suggesting that men process multi-digit numbers in a more holistic fashion (whole numbers), while women process decade and unit digit magnitudes separately.

Likewise, eye-tracking evidence demonstrated that during face and emotion recognition women fixate more strongly on the eyes, independent of view-point, while men tend to focus their gaze more toward the nose in frontal views and the cheeks in profiles, i.e., the view-specific center-of-gravity (e.g., Saether et al., 2009).

The common denominator across the tasks and strategies described so far, is that the sex specific strategies can be linked to visuospatial attention in that participants either self-report their focus of attention, their focus of attention is actively directed toward particular stimulus aspects via different instructions or the use of different stimulus categories, or their focus of attention is recorded using eye-tracking evidence.

Thereby, male strategies appear to share the common feature of being oriented toward more global stimulus features or aspects of the task, i.e., they can be described as holistic. Female strategies however appear to be oriented toward more local stimulus features and can thus be described as decomposed. Consequently, sex-specific strategy use may be linked to sex differences in attentional focus, an idea which will be pursued in the next chapter.

The global-local dissociation has been studied particularly well in the context of emotional memory. It has repeatedly been demonstrated that men show better memory of the gist of an emotional story, while women better remember the details of an emotional story (e.g., Cahill, 2003; Cahill et al., 2004; Nielsen et al., 2011). Thereby, the gist and the detail refer to aspects of visual scenes, which makes it plausible that the strategies described for emotional memory are also linked to differences in visuo-spatial attention.

It is an interesting question, whether sex-specific strategies in other tasks that cannot as easily be related to visuo-spatial attention, can also be linked to this principle. It has for example repeatedly been demonstrated that during verbal fluency tasks,

men produce larger clusters (series of words that belong to the same semantic or phonological category), while women tend to switch more often between different categories (Weiss et al., 2006). Category size has been used as one indicator of conceptual global-local processing, as described in the next chapter (Darwent et al., 2010).

In summary, sex-specific strategy use has been reported for almost every cognitive task for which sex differences in performance have been reported. For several tasks, these strategies may be generalizable via a principle in visuo-spatial attention with male strategies being oriented toward global stimulus aspects and female strategies being oriented toward local stimulus aspects. Note however, that only few of the studies described above (Nielsen et al., 2011, 2013; Pletzer et al., 2013) controlled for menstrual cycle phase, hormonal contraceptive use or sex hormone levels. In emotional memory for example, the focus on the details of an emotional story in women appears to be particularly enhanced during the luteal cycle phase, when women's estradiol and progesterone levels are high (Nielsen et al., 2011, 2013). Thus, for several of these tasks, a hormonal modulation of sex-specific strategy use remains yet to be established.

GLOBAL OR LOCAL—SEX DIFFERENCES IN ATTENTIONAL FOCUS

Individual differences in global-local processing are well-established (Forster and Dannenberg, 2010a,b). Perceptual global-local processing refers to the tendency to process visual stimuli as a whole or in parts, whereas conceptual global-local processing refers to the tendency to think in more concrete or abstract terms (Darwent et al., 2010).

Perceptual global-local processing is traditionally studied using hierarchical stimuli (Navon paradigm), i.e., a global structure made up of local parts (Navon, 1977). These stimuli allow assessing global and local processing independently of each other and in their interaction, since participants attention can be directed toward either the global or the local level. Thereby, participants are asked to respond if a certain predefined target appears at a specified level. From experiments with this paradigm, the concept of global precedence was developed (Navon, 1977, 1981), which includes among others the observation that reactions to global targets are faster than reactions to local targets (***global advantage***).

If sex differences in cognitive tasks are based on sex differences in attentional focus, these differences should be apparent in a Navon paradigm. Indeed several findings have been published that support this view. Using a divided attention paradigm, a local advantage has been demonstrated in women that was absent in men (Roalf et al., 2006), while in a selective attention paradigm, a global advantage has been demonstrated in men that was absent in women (Razumnikova and Vol'f, 2011). However, these findings have been questioned by a lack of sex differences in global advantage using hierarchical line/shape stimuli (Kimchi et al., 2009) or a similarity judgment task (Basso and Lowery, 2004).

In a recent study designed to resolve inconsistencies between these previous reports, we demonstrated that the reduced global advantage in women is strongly dependent on hormonal status (Pletzer et al., 2014b). Women in their luteal phase (high estradiol and progesterone) showed reduced global advantage in comparison to men, but also in comparison to women in their follicular phase (low estradiol and progesterone) and hormonal contraceptive users. Furthermore, global advantage was positively related to testosterone levels, but negatively to progesterone levels, while no relationship was observed with estradiol levels. Thus, an enhanced focus on the global aspects of hierarchical stimuli is probably facilitated by testosterone, while an enhanced focus on the local aspects of hierarchical stimuli is probably facilitated by progesterone. This may explain why only women in their luteal cycle phase, i.e., women with elevated progesterone levels, showed a reduction in global advantage compared to men.

Conceptual global-local processing is traditionally studied via construal level tasks (Darwent et al., 2010), e.g., asking participants to group a given set of words into categories, whereby category size is used as an indicator of global-local processing. To the best of our knowledge, sex differences have not been as explicitly studied, nor consistently been reported for this task, although certain similarities to the verbal fluency or verbal memory tasks are apparent. However, a link between perceptual and conceptual global-local processing has been proposed (Forster, 2009; Darwent et al., 2010; Forster and Dannenberg, 2010a,b). Thus, it may be worth investigating a possible link of sex differences in strategy selection between visuo-spatial and verbal tasks. There is for example evidence for sex differences in the relation between creativity and perceptual global-local processing (Razumnikova and Vol'f, 2012).

Furthermore, several psychological (e.g., mood) or social (e.g., stereotype threat) factors have been identified that can affect the size of sex differences in cognitive tasks (e.g., Hausmann et al., 2009; Hirnstein et al., 2014). Global-local processing has been linked to mood (Basso et al., 1996) and gender stereotype activation (Anderson, 2011), which suggests that these factors should also been taken into account when establishing a link between sex-specific strategy use and global-local processing across different tasks.

LEFT OR RIGHT/COUPLING OR DECOUPLING—HEMISPHERIC INTERPLAY AS NEURAL SUBSTRATE OF SEX DIFFERENCES?

Results from visual hemifield (e.g., Robertson and Lamb, 1991), EEG (e.g., Johannes et al., 1996) and fMRI studies (Fink et al., 1996) indicate, that the right hemisphere shows an advantage for processing of the global level, while the left hemisphere shows an advantage for processing of the local level.

Hemispheric asymmetries have also been assessed for several of the cognitive functions discussed above as being subject to sex-specific strategy use (for reviews see e.g., Wada, 2009; Renteria, 2012). For example, verbal functions appear to be left-lateralized, while visuospatial functions appear to be right-lateralized. Such a lateralization of brain functions is mostly assumed to rely on inter-hemispheric inhibition (Chiarello and Maxfield, 1996), with the hemisphere dominant for a task inhibiting the non-dominant hemisphere. Since a right-hemispheric dominance for visuo-spatial attention has been reported (Heilman and Van Den Abell, 1980; Heilman et al., 1983), the lateralization of global-local processing may explain the general observation of a global advantage. The dominant right hemisphere is responsible for

global processing and inhibits the non-dominant left hemisphere responsible for local processing.

Furthermore, sex differences in brain lateralization have received much attention. Some authors argue that hemispheric asymmetries are more pronounced in men than in women and that a stronger variation in hemispheric asymmetries is apparent in women as compared to men (for reviews see McGlone, 1980; Hausmann and Bayer, 2010; Renteria, 2012). However, a meta-analysis by Voyer (Voyer et al., 2012) suggests that this idea may not hold across tasks and that sex differences in the lateralization of verbal and visuo-spatial functions are modulated by modality (visual vs. auditory). Hausmann and Bayer (2010) argue that sex differences in hemispheric asymmetries may be modulated by intra- and inter-individual variations in sex hormone levels. A menstrual cycle dependent modulation of lateralization may explain the stronger variation in hemispheric asymmetries within the female group (Hausmann and Bayer, 2010).

In that respect it has been demonstrated that the lateralization of brain functions is particularly reduced during the luteal cycle phase, when a woman's estradiol and progesterone levels are high (Hausmann and Gunturkun, 2000). This reduction in hemispheric lateralization has originally been attributed to a progesterone-mediated reduction in inter-hemispheric inhibition, termed ***progesterone-mediated inter-hemispheric decoupling*** (Hausmann and Gunturkun, 2000). Therefore, we hypothesized that the reduction in global advantage we observed during the luteal cycle phase (Pletzer et al., 2014b), was mediated via the progesterone-dependent reduction of inter-hemispheric inhibition during global-local processing. However, recent studies stress the role of estradiol in modulating inter-hemispheric communication (Hausmann and Gunturkun, 2000; Weis et al., 2008; Weis and Hausmann, 2010; Hausmann et al., 2013) over the menstrual cycle and this theory has recently been extended to include sex hormone modulations of inter-hemispheric excitation and integration (Bayer et al., 2008). A relationship between global advantage and estradiol was not observed in our previous study (Pletzer et al., 2014b). A reduction of inter-hemispheric inhibition during high-hormone phases has been demonstrated in visual hemifield (Hausmann and Gunturkun, 2000), and fMRI experiments (Weis et al., 2008). In line with this idea, sex-specific hemispheric specialization has recently been demonstrated during global-local processing in a Navon paradigm (Lee et al., 2012), as well as during emotional memory (Cahill, 2007).

Other theories stress, that some sex hormones may enhance the functioning and intra-hemispheric integration of a particular hemisphere (e.g., Hampson, 1990). It has e.g., been proposed that the "female" sex hormone estrogen enhances left-hemisphere functioning, while the "male" sex hormone testosterone has been discussed to enhance right-hemisphere functioning (e.g., Toga and Thompson, 2003). The latter view is in line with our observation that high levels of testosterone are associated with enhanced global advantage.

While a complete picture of how sex hormones interact with inter-hemispheric communication has yet to emerge, several results indicate that sex hormones modulate inter-hemispheric communication (see Hausmann and Bayer, 2010 for a review). We hypothesize that via this modulation, sex hormones affect global and local attention, which may relate to cognitive strategies in several cognitive tasks. To establish a more complete picture on the sex hormonal modulation of lateralization and the link between lateralization and cognitive strategies, hemispheric asymmetries should be taken into account when studying sex differences in cognitive strategies.

CONCLUSIONS

In summary, it has been found, that sex specific strategies share several features over different cognitive tasks and can be described as global/holistic in men and local/decomposed in women and linked to sex-differences in global-local processing. I hypothesized that sex differences in the lateralization of brain functions accompany these strategies as a result of sex hormone modulation of transcallosal neurotransmission. Empirical evidence linking strategy to lateralization and demonstrating hormone-dependent modulation of strategies as well as lateralization is still lacking for several of the tasks described. This shifts the emphasis from a descriptive comparison of men and women to the question how sex hormones modulate cognition as a whole and can only be answered by an adequate understanding of the changes occurring over the course of the menstrual cycle. The idea of the corpus callosum, gating attentional focus and thereby guiding strategy use in a variety of cognitive tasks represents a perspective toward a link between sex differences in different cognitive tasks.

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Asymmetry of cerebral gray and white matter and structural volumes in relation to sex hormones and chromosomes

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Whilst many studies show sex differences in cerebral asymmetry, their mechanisms are still unknown. This report describes the potential impact of sex hormones and sex chromosomes by comparing MR data from 39 male and 47 female controls and 33 men with an extra X-chromosome (47,XXY).

Methods: Regional asymmetry in gray and white matter volumes (GMV and WMV) was calculated using voxel based moprhometry (SPM5), by contrasting the unflipped and flipped individual GMV and WMV images. In addition, structural volumes were calculated for the thalamus, caudate, putamen, amygdala, and hippocampus, using the FreeSurfer software. Effects of plasma testosterone and estrogen on the GMV and WMV, as well on the right/left ratios of the subcortical volumes were tested by multi-regression analysis.

Results: All three groups showed a leftward asymmetry in the motor cortex and the planum temporale, and a rightward asymmetry of the middle occipital cortex. Both asymmetries were more pronounced in 46,XY males than 46,XX females and 47,XXY males, and were positively correlated with testosterone levels. There was also a rightward asymmetry of the vermis and leftward GMV asymmetry in the cerebellar hemispheres in all groups. Notably, cerebellar asymmetries were larger in 46,XX females and 47,XXY males, but were not related to sex hormone levels. No asymmetry differences between 46,XX females and 47,XXY males, and no overall effects of brain size were detected.

Conclusion: The asymmetry in the planum temporale area and the occipital cortex seem related to processes associated with testosterone, whereas the observed cerebellar asymmetries suggest a link with X-chromosome escapee genes. Sex differences in cerebral asymmetry are moderated by sex hormones and X-chromosome genes, in a regionally differentiated manner.

Keywords: cerebral asymmetry, X-chromosome, sex hormone, gender, MRI

INTRODUCTION

Laterality of the human cerebral hemispheres has profound implications for higher cognitive functions and behavior making both functional and structural asymmetries of the brain utterly intriguing (Toga and Thompson, 2003).

Numerous studies have revealed the consistent presence of functional asymmetries and some of these seem to correspond with structural asymmetries (Toga and Thompson, 2003; Narr et al., 2007). Among the structural asymmetries generally found in adults are a larger right hemisphere than left, which mainly seems to be attributed to white matter volume (WMV), and fronto-occipital asymmetry (torque), where the right prefrontal cortex gray matter volume (GMV) is greater than the left, and the left occipital GMV is greater than the right (Good et al., 2001; Raz et al., 2001, 2004; Hamilton et al., 2007; Rezaie et al., 2009). Computed tomography and magnetic resonance imaging (MRI) studies have shown that these asymmetries are more prominent in right-handers (Bullmore et al., 1995; Hamilton et al., 2007). Cerebral tissue asymmetry has been detected at birth and even as early as at 22 weeks of gestational age (Chi et al., 1977; Hering-Hanit et al., 2001), and is, thus, an early phenomenon in the development of the human brain.

Regional structural and functional asymmetries were initially primarily described for language networks with the striking structural asymmetry of the inferior frontal gyrus (Broca's area) and the planum temporale (Wernicke's area) (Geschwind and Levitsky, 1968; Galaburda et al., 1990). Subsequently, with the application of MRI, several other regional asymmetries have been recognized. For example, left > right asymmetry has been reported in the precentral gyrus, and right > left asymmetry in the cingulate sulcus, the uncus, the anterior insular cortex, the superior temporal sulcus, the caudate nucleus, and the dorsal thalamus (Good et al., 2001; Watkins et al., 2001; Luders et al., 2004; Herve et al., 2005, 2006, 2009). In addition, left > right asymmetry has been found in the putamen, and right > left asymmetry in the hippocampus (Raz et al., 2001, 2004); see also Hou et al. for a

comprehensive review (Hou et al., 2013). Some of the aforementioned asymmetries (hippocampus, planum temporale) exist in non-human primates as well (Lyn et al., 2011); they also seem to be independent of the intracranial volume (Barrick et al., 2005) and are believed to reflect adaptation to a challenging environment. Despite rather extensive research, little is known about their underlying mechanisms. Unraveling these mechanisms is both clinically and theoretically important, as many higher cognitive functions are organized along the left/right axis, and a number of developmental disorders (e.g., dyslexia, autism, schizophrenia, ADHD) are associated with reductions of the normal structural asymmetry (Guerguerian and Lewine, 1998; Schulte-Korne et al., 1999; Irle et al., 2005; Shaw et al., 2009). Recent data from subjects with posttraumatic stress disorder (PTSD) suggest that this reduction may also occur in response to stress (Irle et al., 2005; Kim et al., 2012). Changes in cerebral symmetry may, thus, lead to abnormal functional organization and impaired functioning (Toga and Thompson, 2003).

In the quest to discover the biological underpinnings of cerebral asymmetry, important information may potentially be extracted from studies of sex differences. Right > left hemispheric asymmetry is, according to several reports, more pronounced in adult males than females (Nopoulos et al., 2000; Savic and Lindstrom, 2008). A right > left asymmetry has more consistently been reported in the hippocampus volume (Giedd et al., 1996; Pfluger et al., 1999; Toga and Thompson, 2003; Gilmore et al., 2007; Savic and Arver, 2011) and seems detectable as early as the human fetus stage (de Lacoste et al., 1991)—although there are also studies that failed to detect such differences (Hering-Hanit et al., 2001). There are also reports of sex differences in structural asymmetry of the cerebellum (Fan et al., 2010; Tiemeier et al., 2010).

Sexual dimorphism exhibited in brain asymmetry may be related to the effects of sex hormones, sex chromosome genes, environmental factors, or a combination of the three. Based on animal studies, it has long been believed that cerebral sex differences are linked to prenatal androgen levels and the post-pubertal androgen surges (Giedd et al., 2006; van Rijn et al., 2008; Rezaie et al., 2009; Steinman et al., 2009). The androgen theory, as the sole explanation to structural asymmetries of the brain, has been challenged by findings of asymmetrical expression in some genes of the human embryonic cortex after only 12 weeks of gestation (Sun et al., 2005), that is, before the onset of gonadal production of sex hormones. That genetic programs may also be operating in the development of asymmetry is suggested by studies of patients with sex chromosome aneuploidy, showing left hemisphere atrophy in language processing regions in men with Klienefelter's syndrome (47, X46 XY men) and right parietal lobe atrophy in visuospatial processing regions in women with Turner's syndrome (X0 women) (Baron-Cohen, 2004; Lentini et al., 2013; Savic and Arver, 2013). Subjects with X-chromosome aneuploidy have also shown changes in functional lateralization during language tasks, and a reduced activation of the left superior temporal gyrus and the supramarginal gyrus has been observed in 47,XXY men (van Rijn et al., 2008). A study of 47,XXY men also showed larger right > left asymmetries with regard to non-verbal right-hemispheric tasks (Netley and Rovet, 1984). Furthermore, a more recent study including subjects with Turner's as well as Klienefelter's syndrome suggests that the number of sex chromosomes influences the development of brain asymmetry in a differentiated manner along the antero-posterior axis (Rezaie et al., 2009).

The exact patterns of asymmetry in the gray and white matter of separate brain structures along the antero-posterior axis, however, have not previously been investigated exploratively, and it is possible that both structural and functional asymmetries of some structures could be attributable to sex chromosome-linked processes, whereas others could be affected by sex hormone levels. The present study was designed to address this issue by comparing MRI data from 46,XX females, 46,XY males, and men with Klinefelter's syndrome, 47,XXY. In a recent study, this type of experimental design was shown to be suitable for investigations of the possible effects of sex hormones and sex chromosomes on cerebral tissue (Lentini et al., 2013; Savic and Arver, 2013). Based on our previous reports, which showed several structural asymmetries among healthy controls (Savic and Lindstrom, 2008; Savic and Arver, 2011), special interest has been paid in the present study to the asymmetry of the structural volumes of the caudate, putamen, thalamus, amygdala and hippocampus, and the possible asymmetry of the entire hemispheric volumes. Both voxel-brain morphometry and structural volumetry have been carried out, and blood samples were drawn for analyses of bioactive testosterone and estradiol. The study also includes measurements of the length of the second and fourth digit, known as the 2D:4D ratio. According to several scientific reports, this ratio, especially on the right hand, may serve as a proxy for fetal testosterone (Manning et al., 1998, 2004; Williams et al., 2000; Lutchmaya et al., 2004; Coates et al., 2009; Honekopp and Watson, 2010). A more detailed reasoning for use of the 2D:4D ratio is described in some of our previous studies (Lentini et al., 2013; Savic and Arver, 2013).

Men with Klinefelter's syndrome are born with one or more extra X-chromosomes. Their phenotype is characterized by hypogonadism. Testosterone levels in men with Klinefelter's syndrome are usually normal or subnormal during the prenatal period, but become significantly reduced in relation to control boys during puberty (Carson et al., 1982; Ratcliffe et al., 1994; Niznikiewicz et al., 2000; Aydin et al., 2005; Aksglaede et al., 2006). 47,XXY males are heterosexual, have male identity, and their gender roles (van Rijn et al., 2008).

It is well known that in the somatic cells of 46,XX females, one of the two X-chromosomes is randomly inactivated. However, about 15% of X-linked genes (so called escapee genes) escape this process and will be expressed from both X-chromosomes. Because only a few of the escapee genes have homologs on the Y-chromosome (Ashburner and Friston, 1999; Xu et al., 2002), a major portion of these X-linked genes will be expressed in excess in 46,XX females. They could, thus, play an important role in sexual differentiation.

Based on this information, it is reasonable to expect differences in regional asymmetries between 47,XXY males and male controls. As discussed in our earlier publications (Lentini et al., 2013) these differences could be primarily related to two types of genetic mechanisms.

- In 47,XXY males there could be an excessive expression of genes that lie in the pseudoautosomal regions of the X-chromosome. Those X-escapee genes that have active Y-chromosome homologs will be expressed in a higher dose in 46,XXY males than in 46,XX and 46,XY controls, potentially leading to differences in cortical thickness in *relation to both control groups*.
- Second, because 47,XXY males may also have X-escapee genes that do not have Y-chromosome analogues (Vawter et al., 2007), these genes will be expressed in excess in 47,XXY males only in relation to 46,XY males but *not in relation to 46,XX females.*

Regional asymmetry was first compared between male and female controls. Next, correlation analyses were carried out amongst the controls with respect to asymmetry ratios (right vs. left) and the z-transformed estrogen and testosterone levels, as described previously (Lentini et al., 2013; Savic and Arver, 2013). Finally, it was investigated whether the regional asymmetry in 47,XXY males differed from those of 46,XY males and 46,XX females, and whether 47,XXY males exhibited a same sex, opposite sex, or entirely singular pattern of asymmetry (different from both control groups).

One hypothesis was that 46,XY males would have more pronounced asymmetries than 46,XX females and that this difference, at least in some parts of the brain, could be related to testosterone levels. A further hypothesis was that 47,XXY males would have less pronounced asymmetries than 46,XY males and perhaps also 46,XX females. The underlying rationale was based on the previous observation that 47,X XY males have reduced left lateralization in dichotic listening tasks (Kompus et al., 2011) and on the finding of reduced leftward lateralization (measured with fMRI) during a language task in this population (van Rijn et al., 2008).

It was further hypothesized that asymmetries that were not linked to sex hormone levels among the controls, and that were also similar to those of 46,XX females and 47,XXY males but different from those of 46,XY males, could be related to genes located on the extra X-chromosome, the so-called escapee genes, which do not have Y-chromosome homologs. Furthermore, those asymmetries that were different in 46,XXY males in comparison with both control groups would indicate processes related to the X-escapee genes, which have Y-chromosome homologs; they would, thus, be associated with trisomy (three sex chromosomes), or represent downstream effects of this type of sex-chromosome aneuploidy.

MATERIALS AND METHODS

POPULATION

The population consisted of 33 47,XXY males (age 39 ± 11 years, range 21–50, education 13 ± 3 years), 39 46,XY males (age 35 ± 7 years, range 25–50, education 16 ± 2 years), and 47 46,XX females (age 35 ± 7 years, range 22–50, education 16 ± 2 years), and has also partly been described in our previous studies (Lentini et al., 2013; Savic and Arver, 2013). Due to poor MRI image quality in two subjects the analysis included imaging data from 31 47,XXY males. The 47,XXYmales were recruited from the Center of Andrology, Department of Medicine, Karolinska University Hospital, Sweden, the controls from the general public. The age range was 20–50 years, and all participants were right-handed (Edinburgh Handedness Inventory, Oldfield, 1971; Kinsey et al., 2003), and heterosexual (scored Kinsey 0) according to Kinsey's Heterosexual/Homosexual Scale (Kinsey et al., 2003).

Exclusion criteria included having karyotypic mosaicism (assessing the metaphase chromosomes in cells derived from whole blood according to standard procedure), and having a heredity for, history of, or current psychosis. Furthermore, we excluded subjects with a neurological disease, ADHD, personality disorder, major or bipolar depression, alcohol or substance abuse problems. Mild dyslexia was present in about 60% of the 47, XXY men, but no controls.

Two of the 47,XXY males were diagnosed as adults during the course of infertility investigation, the others were diagnosed in early adolescence. All but two (who were deemed to not need testosterone) had been receiving testosterone supplementation subsequent to the diagnosis and were in treatment at the time of the study. The starting age of testosterone treatment ranged from 15 to 40 years. The Ethics Committee of the Karolinska Institutet Stockholm approved the study, and prior to study all participants signed informed consent forms.

FINGER RATIOS

The 2D:4D ratios of both hands were measured using a steel vernier caliper. Measurements were carried out directly on the fingers, on the ventral side of the hand between the basal crease and the fingertip (Manning et al., 1998, 2004; Lutchmaya et al., 2004; Manno, 2008; Ciumas et al., 2009). As in our earlier study (Savic and Arver, 2013) the 2D:4D ratios of 15 subjects were independently measured by two raters, and the inter-rater correlation was calculated with linear regression (Pearson's coefficient, $p < 0.05$).

VENOUS BLOOD SAMPLES

Venous blood samples were collected from all the subjects between 8 and 10 a.m. in the morning. Plasma testosterone levels, (nmol/L), 17β-estradiol (pmol/L) (radioimmunoassay, Testosterone RIA DSL-4000, Diagnostic Systems Laboratory Inc., TX), and the sex hormone binding globulin (SHBG) were analyzed in the Chemical Diagnostics Laboratory at the Karolinska University Hospital. The levels of bioavailable testosterone (nmol/L) were calculated using an equation developed by Sodergard et al. (1982). Before conducting the statistical analyses of possible correlations between hormone levels and regional asymmetry, the individual levels of 17β-estradiol and bioactive testosterone were z-transformed within each sex group, because natural sex differences in plasma testosterone levels and 17β-estradiol (pmol/L) could lead to false correlations—considering that the respective hormone values would be located at the two extremes of the correlation slope.

MRI

MRI data acquisition

All the subjects were investigated in a whole-body 1.5-Tesla MRI medical scanner (General Electric, Milwaukee, Wisconsin)

equipped with 8-channel phased array receiving. The MRI protocol included the following scans: (1) 3D-weighted T1 SPGR images with 1 mm isotropic voxel size according to a previously described protocol (Ciumas et al., 2010); (2) 2D T2-weighted fast spin echo (FSE) images in the axial plane (effective $TE = 56$ ms, $TR = 2500$ ms, FOV = 24 cm, 23 slices of 3 mm thickness). The latter images were not used in the present report.

Voxel-based morphometry

Voxel-based morphometry (Ashburner, 2007) was performed using the Gaser Toolbox (http://dbm.neuro.uni-jena.de/vbm/) with SPM 5 (The Wellcome Department of Imaging Neuroscience, University College London; www.fil.ion.ucl.ac.uk/spm/) and Matlab 7.3 (Math Works, Natick, MA), as described in several related studies, and using The Diffeomorphic Anatomical Registration Through Lie Algebra toolbox (DARTEL, Wellcome Department of Imaging Neuroscience, University College London, UK; http://www.fil.ion.ucl.ac.uk/spm). All the segmented were modulated (multiplied) by the Jacobian determinants, which allowed direct comparisons of regional differences in the volume of each tissue type.

All the T1-weighted MR images were segmented into gray matter (GM) and white matter (WM), and CSF. The segmented images were then flipped vertically in the midsagittal plane ($x = 0$). Next, both the original and the flipped images were imported to DARTEL to create templates (GM and WM). The GM and WM templates of the entire study group were then normalized to MNI space using the 12-parameter transformation and then flipped vertically in the midsagittal plane ($x = 0$), and a mean image of the original and of the flipped GM templates was created (a mean symmetrical template). The individual images were finally normalized to the symmetrical template and finally smoothed with a 8 mm FWHM kernel.

Within-group asymmetries in regional GMV and WMV were tested through paired t-tests for each group (46,XX, 46,XY and 47,XXY), comparing the individual, MNI normalized, and segmented GM and WM unflipped images with the corresponding flipped images. Significant clusters were calculated for each subject group with peak threshold at $p = 0.001$, FDR corrected at $p < 0.01$ (the more restrictive significance level was used to avoid large confluent clusters).

Between-group asymmetries were tested with a full-factorial design using each group's (46,XX, 46,XY and 47,XXY) unflipped and flipped normalized images as the factor of variance, and using age and total tissue volume (TV) as the covariates of no interest (peak threshold at $p = 0.001$, FDR corrected at $p < 0.05$). TV was calculated as the sum of individual WMV and GMV. The following contrasts were investigated:

$$(46, \mathrm{XX}_{\mathrm{unflipped}} - 46, \mathrm{XX}_{\mathrm{flipped}}) - (46, \mathrm{XY}_{\mathrm{unflipped}} - 46, \mathrm{XY}_{\mathrm{flipped}})$$

$$(46, \mathrm{XX}_{\mathrm{unflipped}} - 46, \mathrm{XX}_{\mathrm{flipped}}) - (47, \mathrm{XXY}_{\mathrm{unflipped}} - 47, \mathrm{XXY}_{\mathrm{flipped}})$$

$$(46, \mathrm{XX}_{\mathrm{unflipped}} - 46, \mathrm{XX}_{\mathrm{flipped}}) - (46, \mathrm{XY}_{\mathrm{unflipped}} - 46, \mathrm{XY}_{\mathrm{flipped}})$$

Each contrast was also run in the opposite direction.

To investigate possible commonalities between two groups vs. the third, conjunctional analyses was used. The following conjunctions were investigated: ($46,\mathrm{XX}_{\mathrm{unflipped\text{-}flipped}} - 46,\mathrm{XY}_{\mathrm{unflipped\text{-}flipped}}$) and ($47,\mathrm{XXY}_{\mathrm{unflipped\text{-}flipped}} - 46, \mathrm{XY}_{\mathrm{unflipped\text{-}flipped}}$); ($46,\mathrm{XY}_{\mathrm{unflipped\text{-}flipped}} - 46,\mathrm{XX}_{\mathrm{unflipped\text{-}flipped}}$) and ($47,\mathrm{XXY}_{\mathrm{unflipped\text{-}flipped}} - 46, \mathrm{XX}_{\mathrm{unflipped\text{-}flipped}}$); ($46,\mathrm{XX}_{\mathrm{unflipped\text{-}flipped}} - 47,\mathrm{XXY}_{\mathrm{unflipped\text{-}flipped}}$) and ($46, \mathrm{XY}_{\mathrm{unflipped\text{-}flipped}} - 47,\mathrm{XXY}_{\mathrm{unflipped\text{-}flipped}}$). Based on previous observations from a similar population, it was assumed that the pattern and degree of asymmetry would be similar among 46,XX females and 47,XXY males and different from that 46,XY males. Therefore, the threshold level of $p < 0.05$ uncorrected was used when testing conjunction ($46,\mathrm{XX}_{\mathrm{unflipped\text{-}flipped}} - 46,\mathrm{XY}_{\mathrm{unflipped\text{-}flipped}}$) and ($47,\mathrm{XXY}_{\mathrm{ůnflipped\text{-}flipped}} - 46,\mathrm{XY}_{\mathrm{unflipped\text{-}flipped}}$), and $p < 0.05$ corrected was used for the remaining two conjunctional tests.

Possible asymmetry correlations with bioactive z-testosterone, z-estradiol, and right hand 2D:4D ratios were tested with multiregression analyses using both control populations, employing age and individual tissue volumes as the covariates of no interest.

Evaluation of structural volumes. Segmentation generated with FreeSurfer was used to derive volumes of the total intracranial volume and the volumes of five subcortical structures: the amygdala, hippocampus, caudate, putamen, and thalamus [FreeSurfer software (www.surfer.nmr.mgh.harvard.edu), according to standard procedure, Fischl, 2012]. Data on cortical thickness, which were also generated by the FreeSurfer program, partly from these populations, has been presented in a separate manuscript. The method for generation of subcortical volumes has been described in detail previously (Fischl et al., 2002, 2004; Fischl, 2012; Savic and Arver, 2013). The segmentation masks were first registered to original grayscale images. When necessary, manual correction was carried out by a person educated in neuroanatomy (who had no information about the identity of the subjects).

Possible right/left asymmetries were tested for each separate structure with paired t-tests (normally distributed data, $p < 0.05$). Each subcortical volume was divided with the individual intracranial volume, retrieved from the FreeSurfer software. Possible differences in the VOI/total brain volume as well as in the asymmetry index (right/left) of each measured volume between the three groups were tested with One Way ANOVA, after ensuring that the data were normally distributed. Group was used as the between factor ($p < 0.05$). Tukey's statistical test was employed for *post hoc* analyses because the sample sizes were unequal.

Delineation of the right and left cerebral hemispheres. The volumes of interest (VOIs) representing the entire right and left cerebral hemispheres were delineated on every second coronal slice of the individual MR images as described previously (Savic and Lindstrom, 2008). Briefly, cerebral hemispheres were divided at the midline in the coronal plane by a hand-drawn line connecting the measured midpoint of the corpus callosum with the midpoint of the hypothalamus, third ventricle, and cerebral aqueduct (Savic and Lindstrom, 2008). Each hemisphere VOI included the ventricles and excluded cerebellum. The right/left hemisphere ratios were tested for normal distribution using the Shapiro–Wilk

test. Because they were not normally distributed, group differences in the right/left ratio were tested with the Kruskal–Wallis Test ($p < 0.05$), and in cases of significant group difference, the separate groups were further compared with each other using the Mann–Whitney test (no Bonferroni correction was applied when testing the right/left asymmetry between 46,XX and 46,XY controls, as a significant rightward asymmetry was predicted based on the related studies, Savic and Lindstrom, 2008).

Correlational analyses. Possible effects of bioactive testosterone and estrogen and also of right hand 2D:4D ratios on GMV and WMV asymmetry were tested exploratively using VBM, through multivariate linear regression analyses. Z-transformed bioactive testosterone and estradiol and the right hand 2D:4D ratio were used as covariates of interest to examine how the respective correlations with GMV and WMV asymmetry in each voxel differed between groups (one calculation for each factor). Age was employed as the nuisance variable. Based on previous reports about the effects of sex hormones (Bramen et al., 2012; Nguyen et al., 2013; Savic and Arver, 2013), it was hypothesized that correlations would exist between tissue asymmetry and sex hormone levels, and the 2D:4D ratios primarily in regions in which GMV and WMV asymmetry differed between 46,XY males and 46,XX females. The significance level for clusters, which overlapped with those showing differences between 46,XY and 46,XX controls, was therefore $p < 0.05$ uncorrected, whereas a threshold of $p < 0.05$ corrected was employed for the remaining brain regions. Correlation analyses with z-estradiol and z-bioactive testosterone were carried out only among controls, as the corresponding hormone levels in 47,XXY males were biased by testosterone treatment. To the contrary the right hand 2D:4D ratio was used as the covariate in a separate analysis with all three study groups. Possible covariation between asymmetries of the entire hemispheric as well as separate subcortical structural volumes, and z-normalized bioactive testosterone values, estradiol values, as well as the 2D:4D ratios were tested using Pearson partial linear correlation ($p < 0.05$) with age as nuisance variable (SPSS, version 21).

RESULTS

DEMOGRAPHIC DATA AND DIGIT RATIOS

Between-subject One-Way ANOVA showed that there was a significant main effect of group on the number of years of education ($F = 14.1$: $p < 0.001$). According to *post-hoc* analysis (Gabriel's procedure), this difference was constituted by the lower education among in the 47,XXY group compared to both male ($p < 0.001$) and female controls ($p < 0.001$), whereas no difference was detected between male and female controls ($p = 0.91$). There were no group differences in regard to handedness or sexual orientation. No gross anatomical abnormalities were found according to an experienced neuroradiologist.

The digit ratio measurements of the two raters were correlated ($r = 0.93$; $p < 0.001$). The results presented here were based on measurements from rater one, as rater two performed ratings for only a portion of the study group. A significant group difference was found for the 2D:4D ratio of the right hand ($p = 0.015$, $F = 4.5$, $df = 2$) but not the left hand ($p = 0.460$, $F = 0.8$, $df = 2$). 47,XXY males and 46,XX females had higher ratios than 46,XY males, while the ratios between 46,XX females and 46,XY males did not differ (**Table 1**).

WITHIN- AND BETWEEN-GROUP ASYMMETRIES IN REGIONAL GMV AND WMV

The data regarding comparisons of regional asymmetries in GMV and WMV are presented in **Table 2**. In summary, all three groups showed a rightward GMV asymmetry in the anterior cingulate, the superior temporal gyrus, the medial occipital cortex, and the vermis cerebelli. Corresponding asymmetries were found also in WMV, the only exception being that no asymmetry was detected in 47,XXY males in the superior temporal gyrus. There were also leftward asymmetries, which, like the rightward asymmetries, showed a similar distribution amongst all three groups.

Table 1 | Demographic data.

		46,XX females *N* = 47		46,XY males *N* = 39		47,XXY males *N* = 33		
	Unit	**Mean**	***SD***	**Mean**	***SD***	**Mean**	***SD***	***F*-value**
Age	Year	35.3	7.4	35.0	6.9	39.0	10.6	2.9
Education	Year	15.9	2.2	15.8	2.4	12.9	2.6	14.1***
Right D2:D4[†]	Ratio	1.00	0.03	0.97	0.02	0.99	0.04	4.5*
Left D2:D4	Ratio	0.99	0.03	0.98	0.03	0.99	0.04	0.78
Oestradiol (plasma)	pmol/L	150.9	113.0	74.0	28.3	103.3	47.7	7.9***
Testosterone (bioactive)	nmol/L	0.5	0.3	6.1	1.5	11.5	6.8	38.3***
L hemisphere volume	cm^3	526.7	56.8	596.8*	41.6	565.6	49.2	
R hemisphere volume	cm^3	525.1	55.2	599.0*	41.4	567.6	74.6	

F-values from group comparisons (One Way ANOVA).

$^*p < 0.05$; $^{***}p < 0.001$.

Possible differences in digit ratios between the separate groups were calculated with Tukey post hoc test ($p < 0.05$).

[†]Difference between 46,XY and 46,XX, $p = 0.042$.

Difference between 47,XXY and 46,XX, $p = 0.998$.

Difference between 46,XY and 47,XXY, $p = 0.041$.

Table 2 | Within group asymmetries in Gray and White matter volume.

Region	Gray matter volumes			White matter volumes		
	Z level	Size, cm^3	Co-ordinates	Z level	Size, cm^3	Co-ordinates
MALE CONTROLS (RIGHT > LEFT)						
R superior temporal gyrus	7.8	10.0	46 −28 −2	inf	2.9	52 −18 4
R medial occipital lobe (cuneus)	7.4	7.2	8 −60 10	4.7	0.8	19 −50 0
R anterior cingulate	5.9	10.0	0 26 0	inf	5.8	8 40 −8
	6.2		8 26 −16			0 28 12
R cerebellum (vermis)	7.8	7.8	10 −64 −38	6.8	6.2	28 −58 −26
MALE CONTROLS (LEFT > RIGHT)						
L temporo-parietal WM				7.1	2.8	−34 −32 16
L planum temporale + precentral gyrus	5.7	6.4	−38 −24 8	inf	3.2	−32 24 4
L caudate	inf	5.0	−10 6 2			
L lateral occipital lobe (middle occipital gyrus)	6.6	6.2	−32 −96 6	6.7	4.8	−20 −88 −4
L cerebellar hemisphere	inf	28.0	−24 −42 −38	6.0	3.2	−12 −52 −46
FEMALE CONTROLS (RIGHT > LEFT)						
R superior temporal gyrus	7.8	7.4	50 −20 −10	4.1	0.2	52 −16 −8
R medial occipital lobe (cuneus)	7.0	4.8	6 −64 14	6.3	3.3	8 −90 14
R anterior cingulate	5.9	13.0	10 60 8	inf	4.8	6 50 −10
R cerebellum (vermis)	6.6	3.2	14 −62 −34	inf	5.2	26 −58 −26
						0 −69 −34
FEMALE CONTROLS (LEFT > RIGHT)						
Part of precentral gyrus, planum temporale	5.7	7.7	−40 −28 16	7.4	2.4	−36 26 2
						−32 14 6
L parahippocampus	6.3		−22 −32 −4	4.2	0.4	−14 −34 2
L caudate	7.5	3.2	−2 10 2	7.4	3.3	−28 22 −12
L inferior frontal WMV						
L middle occipital gyrus				4.8	6.3	−8 −90 14
L cerebellar hemisphere (semilunar lobes)	7.8	12.0	−24 −42 −48			
XXY (RIGHT > LEFT)						
R superior temporal gyrus	6.3	6.0	46 −28 −4			
R anterior cingulate	6.3	1.0	10 64 2	7.2	7.2	12 48 −10
R occipital cortex (cuneus)	6.6	7.2	8 −58 10	5.7	13.0	22 −54 18
R Cerebellum (vermis)	6.1	4.0	10 −64 −46	7.5	5.2	18 −48 −26
XXY (LEFT < RIGHT)						
L planum temporale + part of the pre and post-central gyrus	6.0	4.9	−42 −22 8	7.5	5.6	−34 −36 10
	5.2	2.0	−26 26 0			−56 −8 26
L caudate	7.2	2.4	−10 10 0			
L parahippocampal gyrus	6.8	9.0	−22 −44 −12			
L inferior frontal gyrus				6.7	2.0	−30 24 −12
L occipital cortex (cuneus)	5.1	1.2	−12 −90 10	5.3	2.8	−8 −90 14
						−14 −88 16
L cerebellar hemisphere, semilunar lobes	6.1	4.0	−40 −56 −20			

Clusters denoting GM and WM volumes calculated with the SPM5 VBM toolbox, using TV and age as covariates of no interest. Significant clusters calculated using voxel threshold at p = 0.001, FWC corrected at p = 0.01. MNI co-ordinates indicate the peak values and the indicated regions coverage of the respective cluster. R, right; L, left; inf, infinite.

These asymmetries were detected in the GMV of the motor cortex (covering parts of the planum temporale) (**Table 2**, **Figure 1**), in the left cerebellar hemisphere (the semilunar lobules), in the caudate, and in the parahippocampus. The WMV asymmetries largely followed those in the GMV (**Table 2**).

Interestingly, although the patterns of asymmetry did not differ between the three groups of subjects, there were several significant differences in the degree of asymmetry (**Table 3**, **Figure 1**):

- The rightward GMV and WMV asymmetry in the cerebellar vermis and in the medial occipital lobe was significantly greater in 46,XY males compared to both 46,XX females and 47,XXY males. The medial occipital lobe difference could be due to the fact that men generally have greater occipital GMV in the right occipital lobe than women (Lentini et al., 2013).
- In contrast, the leftward asymmetry in GMV and WMV in the cerebellar semilunar lobuli/peducles was more pronounced in 46,XX females and 47,XXY males than in 46,XY males. Group

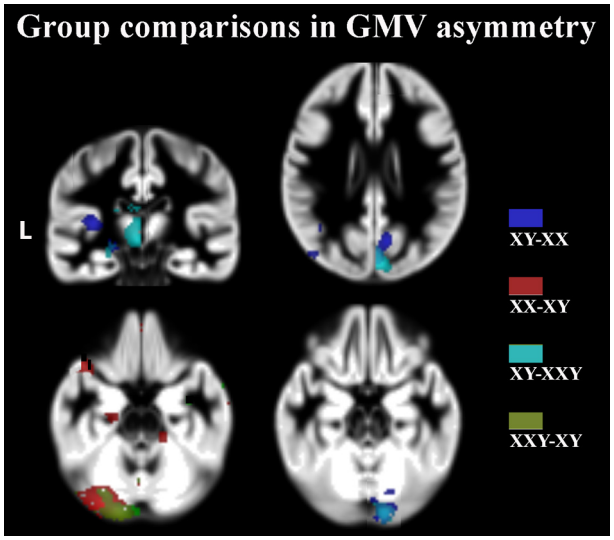

FIGURE 1 | Clusters indicating significant group differences in the GMV asymmetry, calculated using voxel threshold at $p = 0.001$, corrected at $p < 0.05$, FDR. Clusters are superimposed on the symmetric GMV template of the entire study group. L, left side.

differences in cerebellar asymmetries were not constituted by any particular lateralized reduction in one group vs. the other.

- The leftward asymmetry in the GMV of the planum temporale was more prominent in 46,XY males than in 46,XX females, whereas no significant difference was detected in relation to 47,XXY males.

Notably, no differences were found between 46,XX females and 47,XXY males in GMV asymmetry or in WMV asymmetry.

- Finally, conjunctional analyses revealed that the more pronounced leftward cerebellar asymmetry (compared with 46,XY males) was shared between 46,XX females and 47,XXY males. The rightward symmetry in the GMV in the superior temporal gyrus and the GMV of the cerebellar vermis was significantly more pronounced in 46,XY males when compared with both 46,XX females and 47,XXY males. No common clusters were detected with respect to asymmetry of male and female controls in relation to 47,XXY males, nor with respect to both male groups in relation to female controls. Thus, the asymmetry pattern in 47,XXY males was more similar to that of 46,XX females.

Relationships between regional asymmetries in the GMV and WMV, sex hormone values, and digit ratios

The explorative multifactorial regression analysis revealed a significant positive correlation between z-transformed levels of bioactive testosterone and the leftward GMV and WMV asymmetry in the planum temporale/motor cortex ($z = 3.6$ cluster sizes 2.0 cm^3, co-ordinate −42 −8 12 and −40 −42 4 for the GMV, −37 −48 34 for the WMV). Also the rightward asymmetry of the GMV and WMV in the middle occipital lobe was correlated with z-testosterone ($z = 3.5$, cluster sizes 2.0 cm^3, MNI co-ordinate 20 −70 −16 and 8 −62 22 for the GMV and WMV, respectively).

No significant correlations were detected between the 2D:4D ratios and regional asymmetries in the GMV or WMV.

SUBCORTICAL VOLUMES

The volumes, as well as their right/left asymmetry, were normally distributed (Shapiro–Wilk test). Paired *t*-test showed a significant rightward asymmetry in the thalamus in all three groups ($p < 0.0001$ for 47,XXY, $p = 0.043$ for 46,XY and $p = 0.006$ for 46,XX). The putamen volumes, on the other hand, showed a significant leftward asymmetry in all three groups ($p < 0.0001$ in all the groups). Left caudate volume was larger than the right in 46,XY males ($p = 0.016$), whereas no asymmetry was detected in the two other groups. The right hippocampus was larger than the left in 47,XXY males ($p = 0.005$) and 46,XY males ($p = 0.013$), and no hippocampal asymmetry was found in 46,XX females ($p = 0.26$); finally, no asymmetries could be detected in the amygdala volumes in any of the groups. Subcortical values are presented in **Table 4**.

A significant group difference was detected with regard to the asymmetries of the thalamus ($p = 0.001$, $F = 6.9$) and the hippocampus ($p = 0.034$, $F = 3.5$). The rightward thalamus asymmetry was more pronounced in 47,XXY males than in both 46,XY males ($p = 0.013$) and 46,XX females ($p = 0.002$), without any difference among the two control groups. The right > left asymmetry of the hippocampus was significantly greater in 47,XXY males ($p = 0.013$) as well as in 46,XY males ($p = 0.041$) compared to 46,XX females. No asymmetry differences were detected in other structural volumes.

Post hoc analysis: group difference with regard to the relative structural volumes (VOI/TBV)

The ratio of each side's structural VOI to total brain volume (TBV) was calculated *post hoc* to investigate whether the observed group differences in asymmetry were attributed to a difference on either particular side. The VOI/TBV ratios were compared between the groups (One-Way ANOVA, $p < 0.05$ with Tukey's *post hoc* test). Group difference was detected for the left thalamus ($p = 0.001$; $F = 12.2$), as a significantly smaller VOI/TBV ratio was found among 47,XXY males compared to both 46,XY and 46,XX groups ($p < 0.001$ for both). Thus, the greater right/left asymmetry in 47,XXY males was probably due to their reduced left thalamus volume. Other group differences were bilateral. They were constituted by lower relative volumes in 46,XXY men in relation to both control groups for the putamen, ($p < 0.001$; $F = 9.8$ and 10.5), hippocampus ($p < 0.001$, $F = 10.5$ and 15.6 for the right and left side) and the amygdala ($p < 0.001$; $F = 12.1$ and 18.6 for the right and left side, respectively), P was < 0.001, for all the *post hoc* comparisons.

ASYMMETRIES IN THE HEMISPHERIC VOLUMES

The paired-sample *t*-test showed a significant difference (asymmetry) between the right and left hemispheric volumes in the male controls [$t_{(39)} = -2.57$; $p < 0.05$), but not in the female controls [$t_{(47)} = 1.11$; $p = 0.28$] nor in the 46,XXY patients

Table 3 | Between group asymmetries in Gray and White matter volume.

Region	Gray matter volumes			White matter volumes		
	Z level	Size, cm^3	Co-ordinates	Z level	Size, cm^3	Co-ordinates
XY–XX (Right > Left)						
R middle occipital lobe (lingual gyrus)R cerebellum (vermis)	4.3	0.9	14 −90 −2			
	5.0	1.8	6 −68 −48			
R middle frontal gyrus (and WM)	4.3	1.2	26 66 10	4.3	3.2	13 46 −12
R thalamus				5.2	0.5	2 −4 0
XY–XX (Left > Right)						
L planum temporale (and part of inferior motor cortex)	3.7	1.7	−36 −24 10	5.2	0.9	−44 −20 −12
XX–XY (Right > Left)						
XX–XY (Left > Right)						
L cerebellar hemisphere (GM, WM)	5.4	2.0	−32 −70 −28	5.0	2.1	−32 −54 −22
XXY–XX (Right > Left)			–			
XXY–XX (Left > Right)			–			
XXY–XY (Right > Left)			–			
XXY–XY (Left > Right)			–			
L cerebellar hemisphere				3.7	1.7	−38 −68 −32
XY–XXY (Right > Left)						
R cerebellar vermis	3.8	0.4	10 −66 −44	4.0	1.8	16 −60 −26
R middle occipital lobe (cuneus)	4.0	0.3	2 −76 17	4.2	0.4	18 −90 20
XY–XXY (Left > Right)						
L temporal white matter				4.0	1.1	−42 −20 −14
L parahippocampal GM	4.5	2.4	−24 −40 −38			

Clusters denoting GM and WM volumes calculated with the SPM5 VBM toolbox, using TV and age as covariates of no interest. Significant clusters calculated using voxel threshold at p = 0.001, corrected at p < 0.05, FDR. Italics denote trend significance (peak value calculated at T = 0.001, corrected p < 0.1, FDR). MNI co-ordinates indicate the peak values and the indicated regions coverage of the respective cluster. R, right; L, left.

$[t_{(31)} = -1.71;\ p = 0.10]$, (**Table 1**). The Kruskal–Wallis test (the asymmetry of hemispheric volumes was not normally distributed) showed that there was a significant group difference in the right vs. left hemispheric volume ratio (Test statistics 7.8, $df = 2$, $p < 0.019$), (**Table 1**). Subsequent *post hoc* analyses with the Mann–Whitney test showed that this asymmetry was significantly more pronounced in both 47,XXY males ($p = 0.011$) and 46,XY males ($p = 0.025$) than in 46,XX females, whereas no difference was detected between 47,XXY males and 46,XY males ($p = 0.87$).

Relationships between digit ratios, sex hormone levels, and hemispheric and subcortical volumes

Because the distribution of the hemispheric data could not be assumed to be normal, Kendall's tau test, a non-parametric correlation analysis, was used. There was a trend, albeit without a statistical significance, toward an inverse correlation between the digit ratio and the right/left hemispheric ratio ($p = 0.08$, all three groups included), indicating that higher exposure to fetal testosterone was associated with more overall rightward hemispheric asymmetry.

None of the calculated subcortical asymmetries were found to be correlated with z-testosterone, z-estrogen, or right hand digit ratio.

DISCUSSION

The present study was designed to explore possible sex differences in regard to the asymmetry of subcortical structural volumes, and regional gray and white matter volumes. A further purpose was to investigate if and how such asymmetries might be related to sex hormone levels and sex chromosome gene dosage. As in the related studies on the underpinnings of cerebral sex differences, specific comparisons were carried out between 46,XX females, 46 XY males, and 47,XXY males, and included evaluations of possible correlations with sex hormone levels and digit ratios. Asymmetries were detected in all three groups of subjects. They were characterized by greater GMV and WMV in the *right* superior temporal lobe, the cuneus, the anterior cingulate, and cerebellar vermis as well as by a more prominent leftward asymmetry in parts of the precentral gyrus and the planum temporale, and in the left occipital lobe and the cerebellar semilunar lobes (**Table 2**). Similar findings have been reported in several previous investigations (Falk et al., 1991; Steinmetz, 1996; Good et al., 2001; Watkins et al., 2001; Herve et al., 2006; Takao et al., 2011; Saenger et al., 2012), albeit with some variations. The present results provide additional information to the ongoing discussion on the etiology of cerebral asymmetries by showing that 47,XXY males and 46,XX females have several common features in which they differ from 46,XY men, and by suggesting there may be a regionally differentiated involvement of sex hormonal and sex chromosome related factors. In sum, three types of group differences were found: (1) the leftward GMV and WMV asymmetry of the planum temporale and parts of the motor cortex was significantly greater in 46,XY males than in 46,XX females. The degree of this asymmetry correlated with z-transformed testosterone levels. (2) The rightward asymmetry in the medial occipital

Table 4 | Subcortical volumes.

Region	46,XX females *N* = 47	46,XY males *N* = 39	47,XXY males *N* = 31
R Caudate	3.74 ± 0.43	4.21 ± 0.41	3.78 ± 0.43
L Caudate	3.76 ± 0.42	4.27 ± 0.52	3.82 ± 0.43
R Putamen	5.03 ± 0.53	5.56 ± 0.67	4.86 ± 0.49††
L Putamen	5.27 ± 0.52	5.88 ± 0.67	5.17 ± 0.52††
R Hippocampus	3.95 ± 0.42	4.29 ± 0.36	3.87 ± 0.37†††
L Hippocampus	3.93 ± 0.40	4.17 ± 0.45	3.72 ± 0.42†††
R Thalamus	6.97 ± 0.67	7.87 ± 0.79	7.17 ± 0.72
L Thalamus	6.85 ± 0.63	7.69 ± 0.68	6.75 ± 0.68†
R amygdala	1.47 ± 0.13	1.56 ± 0.17	1.38 ± 0.10††††
L amygdala	1.44 ± 0.15	1.55 ± 0.14	1.38 ± 0.13††††

Structural volumes in the respective subject group. Numbers express cm3 (mean and SD).

Reductions in 47,XXY subjects in relation to both control groups: $^{\dagger}p = 0.001$, $F = 12.2$, $^{\dagger\dagger}p < 0.001$, $F = 9.8$ (left) and 10.5 (right). $^{\dagger\dagger\dagger}p < 0.001$, $F = 15.6$ (left) and 10.5 (right) side. $^{\dagger\dagger\dagger\dagger}p < 0.001$; $F = 18.6$ (left) and 12.1 (right) side.

Group difference was detected in the asymmetry of the thalamus ($p = 0.001$, $F = 6.9$) and the hippocampus ($p = 0.034$, $F = 3.5$). The rightward thalamus asymmetry was more pronounced in 47,XXY males than in both 46,XY males ($p = 0.013$) and 46,XX females ($p = 0.002$), without any difference among the two control groups. The right > left asymmetry of the hippocampus was significantly greater in 47,XXY males ($p = 0.013$) as well as in 46,XY males ($p = 0.041$) compared to 46,XX females.

GMV and WMV was more prominent in 46,XY males than in 47,XXY males and 46,XX females, without any difference between the two latter groups. Also, the occipital asymmetry was positively correlated with z-testosterone levels. (3) In 46,XY males there was a more pronounced rightward GMV and WMV asymmetry in the cerebellar vermis and a less pronounced leftward GMV and WMV asymmetry in the lateral cerebellum, without any difference between the two latter groups.

The detected leftward asymmetry of the motor cortex and planum temporale is congruent with the well-known lateralization of language functions, and with the previous reports about sex differences in this asymmetry (Geschwind and Levitsky, 1968; Bear et al., 1986; Amunts et al., 2000; Good et al., 2001; Watkins et al., 2001), although they are sometimes small and vary with age (Hirnstein et al., 2013). The observed correlation with z-transformed testosterone levels provides a potential mechanism for the sexually dimorphic character of these regions. Direct correlation between sex hormone levels in men and women and regional GMV and WMV asymmetries has, to the best of our knowledge, not been tested earlier. A recent study by Lombardo et al., however, reports that fetal testosterone levels can predict the GMV of the left planum temporale (Lombardo et al., 2012). Given this, together with the fact that 2D:4D ratios were elevated among the present 47,XXY group, it is somewhat surprising that no correlation was detected between the right hand 2D:4D ratio and the asymmetry of the planum temporale. In general, it is still unclear through which mechanisms the 2D:4D ratio and prenatal androgen exposure are related (Voracek and Loibl, 2009). Contraru to the body of data discussing fetal testosterone exposure, Knickmeyer et al. (2011) suggested that it may be more appropriate to interpret the 2D:4D ratio in adulthood as an *index of early* testosterone exposure rather than prenatal exposure. The failure to find a correlation with digit ratio in the present study should therefore not be taken as an argument against the influence of fetal testosterone on regional cerebral asymmetry.

The detected cerebellar asymmetries, where a more pronounced *anterior* rightward asymmetry was found in males and a more prominent lateral leftward asymmetry in females, deserve a comment. These were not simply based on lateralized group differences; rather, they seem to reflect asymmetry as a distinct factor. Several previous region-of-interest-based volumetric studies have shown right-greater-than-left anterior volume asymmetry and left-greater-than-right posterior asymmetry in a normal cerebellum (e.g., Lawson et al., 2000; Loeber et al., 2001). One study did not find any significant hemispheric asymmetry, possibly because the method relied on manual tracing of only a few sections (Luft et al., 1998). More directly comparable with the present data are the few studies of structural cerebellar asymmetry carried out with VBM. In a detailed VBM study, Fan et al. found that men had an increased rightward asymmetry within lobules I_IV, IX, and Crus I, and decreased leftward asymmetry within lobules VIIb and Crus II (Fan et al., 2010). That the asymmetry of the anterior cerebellum, which connects to the motorcortex, seems to be more pronounced in males accommodates with the more pronounced leftward asymmetry of the motor cortex in males. The lateral cerebellum, on the other hand, connects to the prefrontal cortex and higher cognitive functions, which according to some reports are associated with a sex-differentiated lateralization (Bolla et al., 2004; Meiron and Lavidor, 2013). Considering that no correlation could be detected with sex hormone levels or digit ratios, and that 47,XXY males and 46,XX females differed in a similar manner from 46,XY males, it would be plausible to assume that the observed group differences in cerebellar asymmetry could be linked to X-chromosome gene expression. While such an assumption would need further testing, it should be noted that asymmetries in regional cerebellar volumes begin to show sex differences early in childhood, although the developmental trajectories differ between boys and girls (Tiemeier et al., 2010). A possible X-chromosome dosage effect is also supported by the finding that certain X-chromosome genes are differentially expressed in the male and female cerebellum (Vawter et al., 2004; Abel et al., 2011), and that the number of sex chromosomes influences the development of brain asymmetry in a complex pattern along the antero-posterior axis (Rezaie et al., 2009).

Unlike the cerebellar and cortical asymmetries, those in subcortical volumes showed no correlation with hormone levels or digit ratios, and no pattern of group differences was found to indicate X-chromosome dosage effects. Differences in relation to 47,XXY males detected in the thalamus could be attributed to the left thalamus atrophy in this group that was detected in the present study and the related previous studies (Savic, 2012; Savic and Arver, 2013). The fact that both male groups had a rightward hippocampus asymmetry that was not detected in females and that did not correlate with sex hormone levels or digit ratios adds a new aspect by raising a question about the possible underlying role of the Y-chromosome. Very preliminary data

on 46,XY females with complete androgen insensitivity shows a more prominent rightward hippocampus asymmetry compared with female controls, which could be taken as an early indication for testosterone-independent Y-chromosome gene effects. The paucity of relevant literature makes it difficult to make more detailed speculations about the possible underpinnings of the observed group differences in hippocampal asymmetry. A more pronounced hippocampal asymmetry in males has also been found in non-human primates (Murphy et al., 1996; Ragbetli et al., 2002; Hou et al., 2013). An early study of mice reports Y-linked influences on the rightward hippocampal asymmetry (van Abeelen et al., 1989), a finding that needs further investigations.

METHODOLOGICAL ASPECTS

The volume of gray matter is mainly affected by the number and size of neurons and glia cells. Asymmetric areas have fewer interhemispheric connections than symmetric areas, perhaps as a result of increased axonal pruning (Galaburda et al., 1990).

Among the more specific issues pertaining to the methods used is that the same structure might have a slightly different spatial location in the two hemispheres.

The effect of between hemisphere differences in spatial homology was reduced by the use of a symmetrical template, linear transformations to this template, and by smoothing of the data, as described previously (Good et al., 2001; Luders et al., 2004). Furthermore, the two hemispheres may differ in shape, primarily due to well-known cerebral anticlockwise torques. By employing twelve-parameter transformations (which include skew in addition to the nine parameters of translation, rotation, and scale), the effects of greater extension of the occipital and frontal poles (petalias) in the left and right hemispheres, and, thus, the torques were reduced. According to a study by Narr et al, sex-related differences in hemispheric shape asymmetry are insignificant (Narr et al., 2007). Nevertheless, it cannot be ruled out that occipital torque, which is reported to be more pronounced in males (Barrick et al., 2005) and less prominent in men with Klinefelter's syndrome (Rezaie et al., 2009), could have influenced the observed occipital differences—although more as a reflection of interrelated differences than due to a methodological bias. Of note is also that the occipital asymmetry in the GMV and WMV was found to be correlated with testosterone levels, even within the 46,XY group.

Another important methodological issue pertains to the interpretation of findings from 47,XXY males. Although androgen levels become notably reduced for these individuals during puberty, when the majority of the subjects started testosterone supplementation, it cannot be excluded that the similarities to 46,XX controls could, to a certain extent, be attributed to early androgen deficits.

The 47,XXY population had significantly lower education, which, theoretically, could have affected the results. However, adding education as nuisance covariate did not change the results.

The fact that hormone levels were, as in several previous studies (Neufang et al., 2009; Peper et al., 2009; Witte et al., 2010), measured on only one occasion is a limitation. Hormone levels vary with activity, stress, and sleeping patterns. Special effort was made to standardize these factors, and it can be claimed that the measures of hormone levels and cerebral GMV and WMV were temporally related (blood samples were taken on the same day as the MRI scans). Admittedly, multiple measurements of serum hormone levels over time might, nevertheless have been more precise for determining the link between circulating hormones and brain morphology.

White matter asymmetries largely followed those of the gray matter, which seems biologically plausible. This observation may, however, also reflect an edge effect—if the interface of one tissue compartment were to be displaced, the difference could be appreciated in both compartments. Such effects were not detected by visual inspection of significance maps.

CONCLUSION

The present study expands the previous neuroimaging literature on cerebral asymmetries by proposing that processes linked to X-chromosome gene dosage affect the pattern of cerebellar asymmetries, whereas processes primarily linked to testosterone levels seem to influence the asymmetry in the planum temporale and part of the motorcortex as well as in the occipital cortex. By identifying brain areas that seem to exhibit the effects of X-chromosome genes, the present results add to the animal data concerning the genes located on X- and Y-chromosomes that could contribute to sex differences in regional asymmetry. Brain asymmetry is usually regarded to be a developmentally adaptive and aiming at improving the computational efficiency by promoting intrahemispheric processes in larger brains (Hutchinson et al., 2003; Abdul-Kareem et al., 2011; Ellis et al., 2013). By showing similarities between 46,XX women and 47,XXY men, whose brains are not smaller than those of 46,XY controls, the present study shows that other factors than cerebral size moderate cerebral asymmetries. Discussion about functional implications is outside the scope of this paper and would require parallel behavioral and fMRI studies. It is, however, important to emphasize that less pronounced asymmetry in women does not imply impaired performance compared to men, but rather a more bilateral hemispheric activation for comparative functions. This emphasizes the importance of comparing males and females separately, especially when trying to make inferences regarding disease-linked changes in hemispheric asymmetry. Whether reduction of natural asymmetry affects men and women similarly in various neuropsychiatric conditions, and how this affects male and female patients with regard to function is, therefore, an issue needing special attention in future studies.

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Interactive effects of culture and sex hormones on the sex role self-concept

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Sex role orientation, i.e., a person's masculinity or femininity, influences cognitive and emotional performance, like biological sex. While it is now widely accepted that sex differences are modulated by the hormonal status of female participants (menstrual cycle, hormonal contraceptive use), the question, whether hormonal status and sex hormones also modulate participants sex role orientation has hardly been addressed previously. The present study assessed sex role orientation and hormonal status as well as sex hormone levels in three samples of participants from two different cultures (Northern American, Middle European). Menstrual cycle phase did not affect participant's masculinity or femininity, but had a significant impact on reference group. While women in their follicular phase (low levels of female sex hormones) determined their masculinity and femininity in reference to men, women in their luteal phase (high levels of female sex hormones) determined their masculinity and femininity in reference to women. Hormonal contraceptive users rated themselves as significantly more feminine and less masculine than naturally cycling women. Furthermore, the impact of biological sex on the factorial structure of sex role orientation as well as the relationship of estrogen to masculinity/femininity was modulated by culture. We conclude that culture and sex hormones interactively affect sex role orientation and hormonal status of participants should be controlled for when assessing masculinity and/or femininity.

Keywords: sex role, menstrual cycle, hormonal contraceptives, sex hormones

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Introduction

Sex role orientation, also referred to as *gender role orientation*, *gender role identity*, *gender role self-concept,* or *gender-related self* has been described as a person's identification with personal attributes that are seen as appropriate for a typical man or woman in a given society, i.e., his or her degree of maleness or femaleness (e.g., Sieverding et al., 2005). These attributes have revealed to be related to various behaviors in numerous psychological domains, as, for example, health (Lefkowitz and Zeldow, 2006; Zimmermann et al., 2011), occupation and work (Sieverding et al., 2005; Garcia-Retamero and Lopez-Zafra, 2009), education (Ritter, 2004; Wolfram et al., 2009; Kessels and Steinmayr, 2013) and cognitive performance (REFs). Some studies demonstrate that sex role orientation was even more influential on outcome measures than biological sex (e.g., Cahill et al., 2004). Consequently, individual differences in sex role orientation, have attracted considerable interest in psychological research since Bem's seminal work (Bem, 1974).

During the past decades it has come into focus, that sex differences are modulated by sex hormone levels and consequently, the hormonal status of female participants, i.e., menstrual cycle phase as well as hormonal contraceptive use. Abilities typically stronger in men than women, like e.g., spatial abilities, are more pronounced in naturally cycling women during their low-hormone follicular phase (Hampson, 1990; Hausmann et al., 2000; McCormick and Teillon, 2001; Dadin et al., 2009). On the contrary, abilities typically stronger in women than in men, like verbal and memory performance are more pronounced in naturally cycling women during their high-hormone luteal phase (Hampson, 1990; Rosenberg and Park, 2002; Dadin et al., 2009). Likewise, hormonal contraceptives display both masculinizing, e.g., enhanced spatial abilities (Wright and Badia, 1999; Wharton et al., 2008), and feminizing, e.g., enhanced verbal and memory abilities (Wright and Badia, 1999; Mordecai et al., 2008), effects on cognitive performance. Furthermore, spatial abilities have been repeatedly related to estradiol and testosterone levels, although there is some discourse about the specific nature of the relationship (e.g., Tan, 2012), while results on the impact of sex hormone levels on verbal- and memory abilities are still inconsistent (e.g., Andreano and Cahill, 2009).

However, studies directly relating sex hormone levels to sex role orientation are rare—mostly focusing on adult and prenatal testosterone (Baucom et al., 1985; Udry, 2000; Al-Ayadhi, 2004), while the question as to whether hormonal status also affects sex role orientation has not been addressed previously. Do women during their follicular phase also perceive themselves as more masculine compared to women during their luteal phase? Do women on hormonal contraceptives actually perceive themselves as more masculine or feminine than naturally cycling women?

These questions tap into the old debate, whether a person's maleness or femaleness, like any other personality characteristics, is determined by socialization or vice versa to which extent it is determined by genetics or other biological factors, like sex hormone levels. With reference to the social role theory, gender roles reflect the traditional social roles of male breadwinners and female caregivers (Eagly and Steffen, 1984; Bosak et al., 2012; Wood and Eagly, 2012). On the other hand, research indicating a role of prenatal androgen exposure for the development of gender-typical behavior endorsed biologically oriented theories (Pasterski et al., 2005; Rammsayer and Troche, 2007; Neave and O'Connor, 2009). At the present, biosocial interaction theories acknowledging causal roles for both biological and social influences on gender related behavior are proposed as more influential (Halpern and Tan, 2001; Eagly and Wood, 2013, p. 1). While the attributes viewed as typical for a man or woman may differ between societies and change over time, genetic factors and sex hormone levels may determine how strongly a person is able to identify herself with those roles. Consequently *gender role stress*, i.e., the amount of stress resulting from the perceived failure to meet gender role standards, has been suggested as one approach to assess gender role identification (Copenhaver and Eisler, 1996).

A number of questionnaires have been developed in order to assess sex role orientation, including the Personal Attributes Questionnaire (PAQ; Spence et al., 1974) and the Bem Sex Role Inventory (BSRI; Bem, 1974). Both questionnaires assess sex role orientation by means of self-ascribed personality characteristics including mainly socially desirable and stereotypical self-perceived personality traits (see Lenney, 1991). These characteristics are assigned to distinct scales for masculinity and femininity. Thus, masculinity and femininity are viewed as different factors (two-component model), rather than viewing the concept of sex role orientation as one bipolar dimension with masculinity and femininity presenting opposite poles of the same concept. While a typical male integrates mostly masculine features and a typical female mostly feminine features, two additional sex-types have been derived from this two-dimensional approach. "*Androgyny*" (Greek: ανήρ, stem ανδρ- *anér-/andr-* = man; γυνή *gyné* = woman) refers to the integration of both masculine and feminine identifications into a person's self-concept, while "*indifference*" refers to the lack of both masculine and feminine identifications. About a third of subjects from Bem's original study display androgyny (Bem, 1974).

Besides the fact that information gained by all self-report measures of gender roles may be biased through introspective limits or strategies of self-presentation (see also van Well et al., 2007), research on the most frequently used measure, the BSRI, revealed several additional flaws. Based on intercultural data it was shown that the questionnaire items did not share its psychometric properties throughout cultures (Sugihara and Katsurada, 1999; Peng, 2006; Colley et al., 2009), and not throughout time (Ballard-Reisch and Elton, 1992). General criticism regarding its proposed factorial structure was raised in a meta-analysis (Choi and Fuqua, 2003). While some factor analyses confirm the validity of the two-dimensional approach after excluding some of the items (Gaudreau, 1977; Waters et al., 1977), others criticize that dimensions differ between men and women (e.g., Pedhazur and Tetenbaum, 1979). Further, it was proposed that the questionnaire may exclude important aspects of gender role, as more aspects than personality traits are linked to gender (Deaux and Lewis, 1984; Athenstaedt, 2003), as, for example, abilities, relationships, physical characteristics, or occupational characteristics (Twenge, 1999).

The present study seeks to address the interplay of societal factors and sex hormone levels in a person's sex role identity and work toward a more universal measure of sex role orientation. Our specific aims are to clarify the following basic questions regarding sex role in a cross-cultural approach:

(a) Does culture or the hormonal status (menstrual cycle, OC use) of female participants affect their sex role orientation or whether they compare themselves to men or women in their sex role ratings?
(b) Is sex role orientation influenced by sex hormone levels, and if so, do sex hormones influence sex role orientation differently in men and women of different hormonal status?
(c) Is the two-component model (masculinity and femininity as separate factors) an adequate description of sex role orientation in men and women of different hormonal status

or is the factorial structure of sex role orientation influenced by sex and hormonal status?

(d) According to what features do participants determine their sex role orientation in different cultures?

Methods

Participants

Data were collected from three samples of participants: (a) an English-speaking Northern American sample of 102 undergraduate students at the University of California, Irvine, comprised of 37 men, 40 naturally cycling women and 35 oral contraceptive (OC) users. (b) A German-speaking Middle European sample of 215 undergraduate students at the University of Salzburg, Austria, comprised of 95 men 67 naturally cycling women and 53 OC users. (c) A German-speaking Middle European Online sample of 308 adult volunteers from the general population, comprised of 73 men, 59 naturally cycling women, 82 OC users and an additional 94 women, who didn't provide us with any information on their hormonal status. The latter were excluded from further analyses. Age of participants is summarized in **Table 1**, cycle data of the naturally cycling groups are summarized in **Table 2**. All participants from samples (a) and (b) were students and had all passed their A-levels (Abitur). Sample (c) was included to also get a picture of sex role orientation from a more general population, which is less homogenous in age and socio-economic status. Although all participants from sample (c) had also passed their A-levels (Abitur), 47% of the men and 36% of the women in the sample were not University students, but in a current working relationship. Only subjects not currently on medication and without psychological, neurological, or endocrine disorders were allowed to participate.

Only naturally cycling women with a cycle length between 20 and 36 days and a variability in cycle length of no more than 7 days were included. These criteria were based on the observations of Fehring et al. (2006). Cycle phase was determined using participants self-reports of their last period date, average cycle length, and—if available—confirmed by follow-up reports of the actual onset of their next period and their estrogen and progesterone levels (see below). Participants were assigned to the early follicular groups with cycle days up to 3 days before ovulation. Participants were assigned to the luteal groups with cycle days from 2 days after ovulation to 2 days before the expected onset of their next period. Ovulation was assumed 14 days before the onset of next period.

TABLE 1 | Age [*M* ± *SD* (years)] of men and women in the three samples.

	Men	Naturally cycling women	OC users
(a) California – PP	21.19 ± 3.13	20.30 ± 1.73	19.96 ± 1.81
(b) Austria – PP	24.56 ± 4.36	23.27 ± 3.80	21.72 ± 3.30
(c) Austria – Online	26.71 ± 10.16	24.63 ± 6.22	22.44 ± 3.35

OC, oral contraceptive; PP, paper and pencil.

Ethics Statement

All participants of samples (a) and (b) gave their signed written consent to participate in the study and approval by the local ethical boards was requested if required [sample (a)]. Sample (c) was an Online sample, i.e., the link to the online questionnaire was sent out to a large number of people, who were free to choose whether to participate by opening the link or not to participate. Participants could terminate the online survey at any time. Only data of those participants who fully completed the online survey were included in the analyses. All methods conform to the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study on sample (a) was approved by the University of California, Irvine's Institutional Review Board. Studies on samples (b) and (c) did not require ethical approval according to the institutional guidelines of the University of Salzburg (Statutes of the University of Salzburg—see https://online.uni-salzburg.at/plus_online/wbMitteilungsblaetter.display?pNr=98160). It is stated in § 163 (1) that ethical approval is necessary for research on human subjects if it affects the physical or psychological integrity, the right for privacy or other important rights or interests of the subjects or their dependents. In § 163 (2) it is stated that it is the responsibility of the PI to decide, whether (1) applies to a study or not. Therefore, we did not approach our institutional review board to obtain ethical approval or a waiver for studies on sample (b) and (c). Since it was non-invasive and performed on healthy adult volunteers who gave their informed consent to participate, (1) did not apply.

Sex Role

Sex role was assessed by a simple six item scale. Rather than using a detailed questionnaire of personality or other characteristics that are viewed as typically masculine or feminine in a given culture, participants directly rated their masculinity and femininity, respectively on a scale of 1–9 (**Figure 1**). While this approach has the disadvantage of only representing the dimensions of masculinity and femininity by a single item, it has several advantages. First, the scale can be used in different cultures irrespective of what is perceived as typically masculine or feminine in a given culture. Second, in contrast to a longer questionnaire, the small number of items allows to address differences in how participants perceive themselves not only with respect to the entire population, but also relative to the own and relative to the opposite sex. Thus, masculinity and femininity are each rated 3 times, relative to (other) men, (other) women or the entire population. Third, due to the direct approach and the short duration, the scale can be combined with a qualitative approach by giving participants the opportunity to explain their

TABLE 2 | Cycle data of the naturally cycling groups among the three samples.

	Cycle length	Follicular		Luteal	
	days (*M* ± *SD*)	*N*	day (*M* ± *SD*)	*N*	day (*M* ± *SD*)
(a) California – PP	29.19 ± 3.52	18	7.89 ± 4.27	22	25.59 ± 6.34
(b) Austria – PP	29.72 ± 3.62	36	7.36 ± 3.71	31	24.26 ± 7.91
(c) Austria – Online	29.03 ± 3.22	31	7.06 ± 4.38	28	23.11 ± 4.65

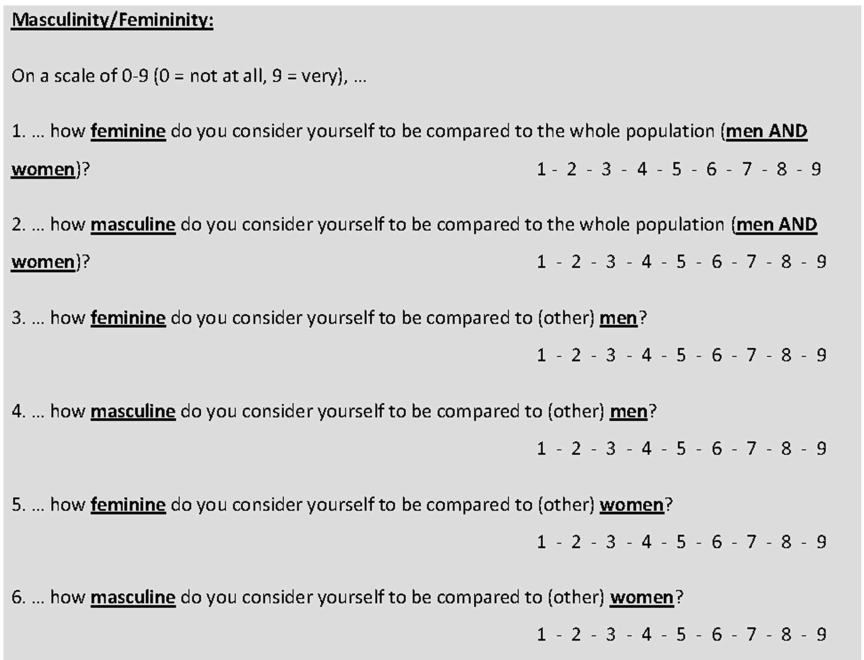

FIGURE 1 | Six item sex role scale (English version).

choices. These explanations can be particularly valuable to assess what is perceived as typically male or female in different cultures and this knowledge can in the long term be used to construct a more comprehensive questionnaire of sex role orientation than is currently available.

Sample (a) completed the English version of the scale, samples (b) and (c) the German version. Samples (a) and (b) completed the scale in a paper- and pencil (PP) design prior to different further experiments, which will not be reported in this manuscript. Sample (c) completed the scale as part of an online questionnaire. Therefore, sample (a) will be referred to as "California – PP," sample (b) as "Austria – PP," sample (c) as "Austria – Online."

Comparable to Bem's (1974) typology, we defined participants with (i) low masculinity as well as low femininity ratings compared to the entire population as *indifferent*, (ii) high masculinity, but low femininity ratings compared to the entire population as *male*, (iii) low masculinity, but high femininity ratings compared to the entire population as *female*, and (iv) high masculinity as well as high femininity ratings compared to the entire population as *androgynous*. Rating larger than five were considered high, ratings lower than or equal to five were considered low. Female participants with a male typology and male participants with a female typology are referred to as *flips*.

In samples (a) and (c) participants were given the opportunity to give a short written explanation for their choice of sex role ratings in their own wording in order to determine the characteristics of their sex role self-concept. This was unfortunately not possible in sample (b) due to time restrictions, which is another reason why sample (c) was additionally included in the study. Reactance statements and circular references to the terms "masculine" or "feminine" were discarded. All other explanations were categorized to one of the following categories by two raters independently:

(i) *Face and Body:* includes statements on their genetically determined bodily appearance, e.g., muscles, body hair, breasts, and facial features.
(ii) *Beauty:* includes statements on the value of make-up, hair styling, fashion, weight control, body building, or any other measure taken to increase one's attractiveness.
(iii) *Activities:* includes statements on activities or interests, participants consider to be typically chosen by men or women, like sports, particular movies, shopping, meeting friends etc.
(iv) *Social contacts:* includes statements on the proportion of males and females in the actual and/or childhood social environment of participants (parents, siblings, friends, coworkers, and other peer relations) as well as how comfortable they feel among men or women.
(v) *Personality:* includes statements on personality traits, participants consider as typically male or female.
(vi) *Emotion:* includes statements on how reflective and open participants are about their emotions and whether or not they like to think or talk about their and other's emotions.
(vii) *Cognition*: includes statements on cognitive skills, participants consider as typically male or female, e.g., orientation in a spatial environment, logics, technology, multi-tasking.

Sex Hormones

Sex hormone levels were quantified from saliva samples in samples (a) and men and naturally cycling women of sample (b) using Salimetrics and DeMediTec ELISA kits, respectively, for Progesterone, 17β-Estradiol, and Testosterone. In men and

naturally cycling women all three hormones were evaluated. In OC-users only 17β-Estradiol and Testosterone were evaluated due to the high variability in synthetic progestins used in OC. Until hormone assessment, saliva samples were stored at −20°C and centrifuged two times at 3000 rpm for 15 and 10 min, respectively. For each participant we calculated the mean level for each hormone over the values assessed in the three samples that were collected over the course of an hour. The value for each sample was determined as the mean over duplicate measurements to ensure reliability of the assessment. Hormone levels with a Coefficient of Variance higher than 25 between duplicate samples were excluded. All hormone levels were within the expected range for the respective participant group.

Statistical Analyses

Statistical analyses were performed using PASW statistics 17.0. Sex role ratings were not expected to be normally distributed over all participants or in any subgroup[1], as of course men were expected to have primarily high masculinity ratings and women were expected to have primarily high femininity ratings. Therefore, non-parametric tests were applied.

To determine, whether the hormonal status of participants affected their sex role ratings, the following analyses were performed. Mann–Whitney *U*-tests were used to compare (i) the sex role ratings of follicular and luteal women, in order to address menstrual cycle-dependent on the one hand and (ii) the sex role ratings of OC users and naturally cycling women to address oral contraceptive-dependent effects on the other hand (see Section Does Culture or Hormonal Status Affect the Sex Role Self-concept? for the results).

To determine, whether the reference group influenced participants sex role ratings, i.e., whether sex role ratings differed depending on whether they were given in comparison to men, women or the entire population, the following analyses were performed (see Section Which Participants Compare themselves more Strongly to Men, Which to Women? for the results):

- Masculinity/femininity ratings in reference to men were compared to masculinity/femininity ratings in reference to women using Wilcoxon signed ranks tests.
- Masculinity/femininity ratings in reference to the entire population were compared to the mean of masculinity/femininity ratings in reference to men and women using Wilcoxon signed ranks tests.

Additionally, to determine, whether men, naturally cycling women and OC users in the different samples compared themselves more strongly to men or women, we used a stepwise multiple regression procedure. Masculinity/femininity ratings in reference to the entire population were entered as dependent variable and masculinity/femininity ratings in reference to men and women were entered as independent variables, to see whether participants overall rating of their masculinity/femininity (i.e., their rating relative to the entire population) depended more strongly on how they perceived themselves relative to men or how they perceived themselves relative to women.

To assess whether sex hormone levels related to sex role ratings, interrelations between the six sex role ratings and the levels of testosterone, estrogen and progesterone were evaluated by Spearman correlations (i) over all participants, (ii) separately for men, follicular women, luteal women, and OC-users (see Section Does the Sex Role Self-concept Relate to Sex Hormone Levels? for the results).

To determine whether the concept of sex role orientation had a one-factorial structure with masculinity and femininity forming opposite poles of one factor or a two-factorial structure with masculinity and femininity loading high on different factors, a principal component analysis was performed using varimax rotation (see Section Do Culture, Sex, or Hormonal Status Affect the Factorial Structure of the Sex Role Self-concept for the results). Due to the small number of factors and observed variables, we did not perform a confirmatory factor analysis.

After examining the factorial structure of sex role, we explore sex role typology among men and women from different cultures by reporting the frequencies of the four typologies described above separately for men, follicular women, luteal women and OC users for each sample and comparing them between groups using χ^2-tests (see Section Typology for the results).

The frequencies of explanations falling into the categories listed above are reported separately for men, follicular women, luteal women, and OC users as well as all participants for each sample and compared between groups and samples using χ^2 tests (see Section Characteristics of the Sex Role Self-concept for the results).

If not specified differently, the sample sizes included in the analyses equaled the description in the participants section.

Results

Does Culture or Hormonal Status Affect the Sex Role Self-concept?

Culture Dependent Effects

We did not observe any differences in masculinity and femininity ratings of men and women between the three samples in Kruskall–Wallis tests (all $\chi^2 < 3.56$, all $p > 0.16$).

Sex Dependent Effects

As expected, in all samples men rated themselves as significantly more masculine and significantly less feminine than women irrespective of the reference group as assessed by Mann–Whitney *U*-Tests (all $Z > 7.19$, all $p < 0.001$, compare **Figure 2**).

Menstrual Cycle Dependent Effects

We did not observe any differences between sex role ratings of follicular and luteal women in any sample (all $Z < 1.70$, all $p > 0.08$, compare **Figure 2**).

OC Dependent Effects

In all three samples, OC users rated themselves significantly more feminine in reference to men than naturally cycling women (all $Z > 1.92$, all $p < 0.05$). In the Austrian Online-sample

[1]This assumption was confirmed by significant results of a Kolmogorov–Smirnov Test for normality for all six variables.

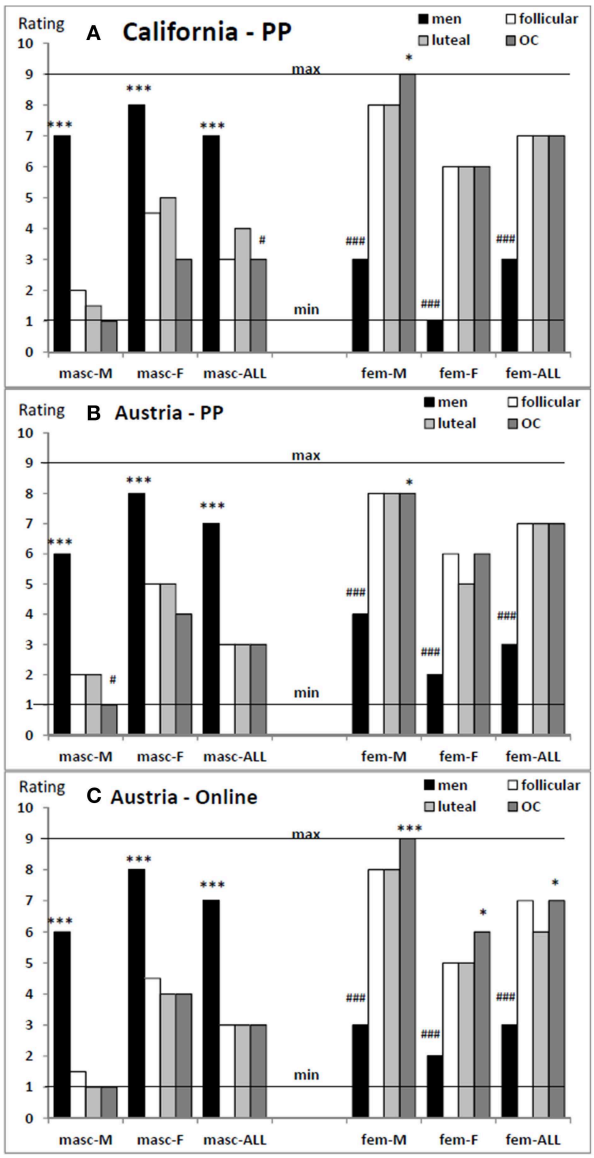

FIGURE 2 | Median of the six sex role ratings in men, follicular women, luteal women and OC users of the three samples. Men rate themselves more masculine and less feminine than women. Follicular and luteal women did not differ in their ratings. OC-users rate themselves more feminine less masculine than naturally cycling women. All groups rate themselves more masculine and less feminine in reference to women than in reference to men. Ratings in reference to the entire population do not equal the mean of ratings in reference to men and women. PP, paper-and-pencil; OC, oral contraceptive; masc, masculine; fem, feminine; M, in reference to men; F, in reference to women; ALL, in reference to the entire population; min, minimum rating; max, maximum rating. Ratings significantly higher than in the other groups: *all $p < 0.05$, **all $p < 0.01$, ***all $p < 0.001$; ratings significantly lower than in the other groups: #all $p < 0.05$, ##all $p < 0.01$, ###all $p < 0.001$. **(A)** California – PP, **(B)** Austria – PP, **(C)** Austria – Online.

drawn from the general population, all three femininity ratings were higher in OC users than in naturally cycling women (all $Z > 2.35$, all $p < 0.05$, compare **Figure 2**). In the California PP undergraduate sample, OC users furthermore, reached lower masculinity ratings than naturally cycling women in reference to the entire population ($Z = 2.02$, $p < 0.05$), while in the Austrian PP undergraduate sample, OC users reached lower masculinity ratings than naturally cycling women in reference to men ($Z = 2.26$, $p < 0.05$).

Which Participants Compare themselves more Strongly to Men, Which to Women?

In all samples all participants rated themselves significantly more masculine in reference to women than in reference to men and significantly more feminine in reference to men than in reference to women (all $Z > 1.95$, all $p < 0.05$, compare **Figure 2**). We observed that in several cases the averaged ratings in reference to men and women deviated significantly from the ratings in reference to the entire population (e.g., men: all $Z > 1.95$, all $p < 0.05$). This observation raised the question, which reference group men and women of different hormonal status use to determine their ratings in reference to the entire population, i.e., which rating (in reference to men or women) has a stronger predictive value for the ratings in reference to the entire population.

Results of stepwise multiple regression are summarized in **Table 3**. In all three samples men determined their sex role largely in reference to men, i.e., they compared themselves more strongly to men than to women. In both undergraduate samples luteal women and OC-users determined their sex role in reference to women, i.e., they compared themselves more strongly to women than to men. However, follicular women determined their sex role largely in reference to men. In the Austrian Online sample drawn from the general population all female groups determined their sex role in reference to women.

Does the Sex Role Self-concept Relate to Sex Hormone Levels?

Sex hormone levels were available from 35 men, 18 follicular women, 21 luteal women, and 33 OC users in the US undergraduate sample, as well as from 39 men, 19 follicular, and 14 luteal women in the Austrian undergraduate sample. Means and Standard deviations for each group are displayed in **Table 4**.

All Participants

In both samples, testosterone correlated positively with all masculinity ratings (all $r > 0.49$, all $p < 0.001$) and negatively with all femininity ratings (all $r < -0.48$, all $p < 0.001$). In the US undergraduate sample, estrogen predicted masculinity ratings negatively (both $r < -0.19$, all $p < 0.05$) and femininity ratings positively (both $r > 0.18$, both $p < 0.06$) in reference to men and women, but not the ratings in reference to the entire population (both $|r| < 0.16$, both $p > 0.10$). These correlations with estrogen did not reach significance in the Austrian sample. In the US undergraduate sample, progesterone predicted all masculinity ratings negatively (all $r < -0.24$, all $p < 0.05$) and all femininity ratings positively (all $r > 0.34$, all $p < 0.01$). In the Austrian undergraduate sample, these correlations with progesterone only reached significance for ratings in reference to men (masc: $r = -0.27$, $p < 0.05$; fem: $r = 0.24$, $p < 0.05$).

TABLE 3 | Results of stepwise multiple regression for men, naturally cycling women and oral contraceptive (OC) users in the three samples.

	Men			Follicular			Luteal			OC		
		β	*t*		β	*t*		β	*t*		β	*t*
CALIFORNIA – PP												
MascM[a]	ENT	0.66	5.16***	ENT	0.81	5.49***	REM	0.04	0.24	E/R	0.46	3.80**
MascF[a]	REM	0.28	1.94	REM	0.34	1.94	ENT	0.78	5.65***	ENT	0.82	6.96***
FemM[b]	ENT	0.82	8.42***	ENT	0.78	4.99***	REM	−0.07	−0.35	REM	−0.04	−0.25
FemF[b]	REM	0.20	1.53	REM	0.32	1.72	ENT	0.87	8.06***	ENT	0.78	6.03***
AUSTRIA – PP												
MascM[a]	ENT	0.75	11.04***	ENT	0.69	5.57***	R/E	0.32	2.47*	REM	0.13	0.92
MascF[a]	R/E	0.32	4.70***	R/E	0.47	4.11***	ENT	0.75	6.09***	ENT	0.68	5.95***
FemM [b]	ENT	0.85	15.61***	R/E	0.34	2.47*	REM	0.24	1.83	REM	0.21	1.53
FemF [b]	R/E	0.25	4.31***	ENT	0.63	4.78***	ENT	0.75	6.18***	ENT	0.49	3.61**
AUSTRIA – ONLINE												
MascM[a]	ENT	0.76	9.76***	R/E	0.48	4.17***	R/E	0.30	2.47*	R/E	0.49	8.37***
MascF [a]	REM	0.19	1.80	ENT	0.67	4.95***	ENT	0.86	9.21***	ENT	0.81	12.39***
FemM [b]	R/E	0.37	3.84***	REM	0.17	1.18	REM	0.23	1.72	R/E	0.32	4.45***
FemF [b]	ENT	0.72	8.70***	ENT	0.69	7.35***	ENT	0.80	7.35***	ENT	0.79	11.45***

MascM, masculinity in reference to men; mascF, masculinity in reference to women; femM, femininity in reference to men; femF, femininity in reference to women.
[a] Tested as predictors of masculinity in reference to the entire population.
[b] Tested as predictors of femininity in reference to the entire population.
**p < 0.05, **p < 0.01, ***p < 0.001, ENT, variable entered by procedure; REM, variable removed by procedure; R/E, removal yields better model, but entry of variable also admissible. For ENT variables the β- and t-values for sole entry are reported, for REM or R/E variables the β- and t-values as if entered are reported. Reference groups were determined from ENT variables and marked light gray.*

TABLE 4 | Hormone levels (*M* ± *SD*).

	Testosterone	Estradiol	Progesterone
CALIFORNIA – PP			
Men ($n = 35$)	156.93 ± 50.47	2.34 ± 0.94[b]**	73.00 ± 50.47[b]***
Follicular ($n = 18$)	74.44 ± 27.93[a]***	2.64 ± 0.89	76.94 ± 37.36[b]**
Luteal ($n = 21$)	64.89 ± 20.39[a]***	3.14 ± 0.98	148.58 ± 109.28
OC ($n = 33$)	49.78 ± 27.77[a]***	2.60 ± 1.01	
AUSTRIA – PP			
Men ($n = 39$)	112.87 ± 47.12	2.63 ± 0.80[b]***	94.21 ± 97.98[b]***
Follicular ($n = 19$)	46.76 ± 19.17[a]***	2.51 ± 0.83[b]***	71.38 ± 74.61[b]***
Luteal ($n = 14$)	53.12 ± 27.27[a]***	2.83 ± 1.48	385.68 ± 382.57

[a] Significantly lower than in men.
[b] Significantly lower than in luteal women.
***p < 0.01, ***p < 0.001.*

In the single group analyses, we found contrary results in the US and Austrian sample.

Men

Estrogen predicted masculinity ratings in reference to the entire population positively ($r = 0.38$, $p < 0.05$) and femininity ratings in reference to the entire population and to men negatively (both $r <$ -0.38, both $p < 0.05$) in US men. The more estrogen, the more masculine and the less feminine US men rate themselves. However, estrogen predicted masculinity ratings in reference to women negatively (r = -0.36, $p < 0.05$), while femininity ratings were unaffected by estrogen in Austrian men. The more estrogen, the less masculine Austrian men they rate themselves. Progesterone and testosterone did not affect sex role ratings in men in any sample (all $r < 0.17$, all $p > 0.29$).

Follicular Women

In follicular women of the US sample, only femininity ratings in reference to men showed an interrelation with sex hormone levels. They were positively predicted by both progesterone ($r = 0.50$, $p < 0.05$) and testosterone ($r = 0.59$, $p < 0.01$), but unrelated to estrogen ($r = 0.18$, $p = 0.48$). The more progesterone and testosterone, the more feminine US follicular women rated themselves in reference to men. In follicular women of the Austrian sample, testosterone and progesterone levels were not significantly related to sex role ratings. However, estrogen levels affected follicular women's sex role ratings in reference to other women. Masculinity ratings were positively predicted by estrogen levels ($r = 0.49$, $p < 0.05$), while femininity ratings were negatively predicted by estrogen levels (r = -0.59, $p < 0.01$). The more estrogen, the more masculine and the less feminine Austrian follicular women rated themselves in reference to other women.

Luteal Women

In luteal women of the US sample, we found estrogen to positively predict femininity ratings (significantly in reference to women: $r = 0.46$, $p < 0.05$; by trend in reference to men: $r = 0.37$, $p = 0.09$). The more estrogen, the more feminine US luteal women rated themselves. In luteal women of the Austrian

sample, no relationship between sex role ratings and sex hormone levels was observed.

Sex hormone levels and sex role were unrelated in OC users of the California PP sample (all $|r| < 0.15$, all $p > 0.30$).

Do Culture, Sex or Hormonal Status Affect the Factorial Structure of the Sex Role Self-concept

Results of Principal Component Analyses over the six sex role ratings in the three samples are summarized in **Table 5**. In the Northern American sample, sex role seems to comprise a one factor structure in men and OC users with masculinity and femininity comprising opposite poles of the same construct, but a 2 factor structure in naturally cycling women with masculinity and femininity items loading high on different factors. In both Middle European samples, sex role seems to comprise a 2 factor structure in men, with masculinity and femininity ratings loading high on different factors and a 1 factor structure in OC users with masculinity and femininity comprising opposite poles of the same construct. In follicular women of the middle European sample, a 2 factorial structure was obtained. However, masculinity and femininity ratings do not completely load on different factors. Reference group seems to be a second determinant of the factors. Luteal women display a one factor solution like OC-users in the PP undergraduate sample and a 2 factorial solution like men in the online sample.

Typology

Table 6 summarizes the frequency of each sex role type for men, follicular women, luteal women, and OC users among the three samples. In the California-PP undergraduate sample and the Austrian online sample drawn from the general population, there was a significantly higher number of indifferent participants relative to androgynous participants among men compared to the female groups (both $\chi^2 > 4.36$, $p < 0.05$), while there was a trend toward a higher number of indifferent participants relative to androgynous participants among women compared to men in the Austrian undergraduate sample ($\chi^2 = 3.12$, $p = 0.08$). The absolute number of indifferent and androgynous participants did not differ between men and women in sample (a) (both $\chi^2 < 2.62$, both $p > 0.10$). In sample (b) there

TABLE 5 | Varimax rotated solution of principal components analysis with open number of factors.

	Men		Follicular		Luteal		OC	
	Factor1	**Factor2**	**Factor1**	**Factor2**	**Factor1**	**Factor2**	**Factor1**	**Factor2**
CALIFORNIA – PP								
mascM	−0.85			0.92	0.85	0.89		
MascF	−0.74			0.82	0.81	0.86		
mascALL	−0.80			0.94	0.78	0.92		
FemM	0.88		0.91		0.81	−0.74		
FemF	0.81		0.84		0.92	−0.87		
femALL	0.89		0.91		0.95	−0.83		
Variance	68.80	10.25	48.25	35.17	62.11	19.34	73.00	11.82
PCA solution	1 component		2 components		2 components		1 component	
AUSTRIA – PP								
mascM	0.83		0.94		0.65	0.72		
MascF	0.80			−0.68	0.83	0.82		
mascALL	0.86		0.76		0.88	0.80		
FemM		0.92	−0.72		−0.70	−0.57		
FemF		0.64		0.92	−0.81	−0.67		
femALL		0.94		0.78	−0.83	−0.70		
Variance	58.66	18.87	53.34	21.35	62.03	16.51	51.35	16.49
PCA solution	2 components		2 components		1 component		1 component	
AUSTRIA – ONLINE								
MascM	0.91			0.93	0.80	0.77		
mascF	0.84		−0.62	0.52	0.94	0.85		
mascALL	0.88			0.87	0.95	0.88		
FemM		0.91	0.93			0.82	−0.75	
femF		0.76	0.67			0.86	−0.80	
FemALL		0.87	0.89			0.78	−0.83	
Variance	60.88	19.91	50.22	26.63	57.86	23.30	66.11	15.68
PCA solution	2 components		2 components		2 components		1 component	

MascM, masculinity in reference to men; mascF, masculinity in reference to women; mascALL, masculinity in reference to the entire population; femM, femininity in reference to men; femF, femininity in reference to women; femALL, femininity in reference to the entire population.

TABLE 6 | Frequency [%] of Typologies among men and women in the three samples.

	Masc	Fem	(a) California – PP				(b) Austria – PP				(c) Austria – online			
			Men	Foll.	Luteal	OC	Men	Foll.	Luteal	OC	Men	Foll.	Luteal	OC
Indiff.	low	low	16.2	11.1	9.1	4.0	10.4	22.2	25.8	18.6	21.9	19.4	14.3	7.3
Male	high	low	78.4	5.6	0.0	4.0	79.2	2.8	3.2	4.7	76.7	3.2	14.3	9.8
Female	low	high	5.4	72.2	81.8	88.0	4.2	69.4	67.7	74.4	0.0	71.0	64.3	74.4
Androgyn	high	High	0.0	11.1	9.1	4.0	6.3	5.6	3.2	2.3	1.4	6.5	7.1	8.5

Foll., follicular; OC, Oral contraceptive; indiff., indifferent; PP, paper and pencil; masc, masculinity; fem, femininity.

was a significantly higher number of women than men among indifferent participants ($\chi^2 = 4.84$, $p < 0.05$), but not among androgynous participants ($\chi^2 = 0.76$, $p = 0.38$). In sample (c) there were by trend more indifferent and less androgynous participants among men compared to women (both $\chi^2 > 3.49$, both $p = 0.06$). Furthermore, there was a significantly higher number ($\chi^2 = 8.57$, $p < 0.01$) of flips among women (female → male) compared to men (male → female) in sample (c), but not in samples (a) and (b) (both $\chi^2 < 0.52$, both $p > 0.47$).

Characteristics of the Sex Role Self-concept

Table 7 summarizes which features are commonly named by participants to explain their masculinity and femininity ratings. The inter-rater agreement was 95%. Only categories on which both raters agreed are listed. In both samples, more women than men gave explanations for their sex role ratings. While Northern American participants named mostly activities and the importance of beauty, fashion and body care as determinants for their sex role ratings, middle European participants referred predominantly to personality traits and only secondarily to activities and the importance of beauty, fashion and body care.

TABLE 7 | Percentage of participants naming features as explanation for their sex role ratings that were categorized to the categories on the left.

	Men	Follicular	Luteal	OC users	Total
CALIFORNIA – PP					
Face and body	0	11	0	0	2
Beauty care	0	28	18	6	10
Activities	5	22	23	12	13
Social contacts	0	17	9	0	4
Personality	5	17	9	3	7
Emotion	3	0	0	3	2
Cognition	0	0	0	3	1
AUSTRIA – ONLINE					
Face and body	4	6	0	7	5
Beauty care	4	12	22	7	9
Activities	7	6	25	7	10
Social contacts	3	6	9	9	6
Personality	18	16	22	21	19
Emotion	4	6	3	0	3
Cognition	1	9	0	2	3

Discussion

The present study assessed self-rated masculinity and femininity in relation to hormonal status and sex hormone levels in participants from different cultures and different educational and socioeconomic status. Influence of culture and hormonal status were investigated on absolute sex role ratings, factorial structure of sex role ratings, reference group and aspects participants rated as relevant to their sex role orientation.

Men Compare themselves to Men, Women to Women, but not during the Follicular Phase

Menstrual cycle does not affect self-perceived masculinity or femininity, even though it has been demonstrated to significantly modulate cognitive sex differences (Hampson, 1990; Hausmann et al., 2000; Rosenberg and Park, 2002). Also only minor differences in the factorial structure of sex role were observed between the cycle phases, as shall be discussed in the following. However, menstrual cycle phase seems to affect which reference group women use when determining their masculinity and femininity scores.

Regression analyses revealed that across cultures, men compare themselves more to men, even when explicitly asked to make comparisons in reference to the entire population. As masculinity ratings in reference to men are naturally lower than masculinity ratings in reference to women, ratings in reference to the entire population may underestimate men's masculinity and/or vice versa overestimate their femininity. Likewise, naturally cycling women during their luteal phase as well as OC users compare themselves to other women and their ratings in reference to the entire population may therefore, underestimate their femininity and/or overestimate their masculinity.

During the follicular cycle phase, however, naturally cycling women, show a tendency to compare themselves to men (US sample) or men and women about equally (Austrian samples). Consequently, their self-ratings in reference to the entire population may be most accurate. Interestingly, in the Austrian samples, follicular women seem to determine their masculinity in reference to men and their femininity in reference to women. This was not only reflected in the regression analyses, but in the factorial structure of their sex role ratings, suggesting two different bipolar dimensions for ratings in reference to men and

ratings in reference to women. This shift in reference group across the menstrual cycle is in good accordance with the shifts from increased spatial (typically male-dominated) to increased verbal (typically female dominated) abilities from the follicular to the luteal phase (e.g., Hampson, 1990; Hausmann et al., 2000; Rosenberg and Park, 2002). A similar shift in absolute sex role ratings might be observable in a within-subjects design to compare the cycle phases rather than the between-subjects design employed in the present study. Consequently, menstrual cycle phase should be taken into account when assessing a person's sex role orientation.

OC-use Alters Sex Role Orientation and Its Factorial Structure

Hormonal contraceptive users rated themselves as significantly more feminine and significantly less masculine than naturally cycling women independent of culture. This effect is striking since both masculinizing and feminizing effects of hormonal contraceptives have been reported in the cognitive domain. However, even though OC users score better in spatial tasks than naturally cycling women (Wright and Badia, 1999; Wharton et al., 2008) and show masculinized brain activation patterns during number processing (Pletzer et al., 2014), they do perceive themselves as less masculine than naturally cycling women irrespective of their cycle phase. The simultaneous perception of reduced masculinity and enhanced femininity leads to a one-factorial structure of sex role in OC-users as opposed to the two-factorial structure mostly observed in naturally cycling women. Consequently, more female and less androgynous, indifferent or flipped to male sex types were observed in OC-users as compared to naturally cycling women. These results are particularly important as they could be replicated across three different samples, from different cultures, different educational, and socioeconomic status and different mode of data collection. It remains to be resolved, how the synthetic progestins and ethinyl-estradiol contained in OC mimic endogenous sex hormone actions to cause this shift in sex role orientation.

The Relationship between Estrogen and Sex Role Orientation Depends on Its Factorial Structure

Correlations of masculinity and femininity ratings with sex hormone levels as observed over all participants are in accordance with sex differences in sex role ratings. Male sex and high testosterone favor high masculinity and low femininity ratings, while female sex and high estrogen and/or progesterone favor high femininity and low masculinity ratings. Importantly, while testosterone and progesterone seem to be more responsible for the modulation of sex role ratings across groups, estrogen seems to modulate sex role ratings within the different hormonal status groups. However, these within-group results are puzzling at first sight. As the differences between the US and Austrian sample in correlations between sex hormones and sex role ratings somehow resemble the differential factorial structure of sex role in men and women between the US and Austrian undergraduate samples, these shall be discussed in parallel.

In the US, undergraduate men perceive masculinity and femininity as two bipolar ends of one *dimension* and their *estrogen levels are positively related to masculinity* (and negatively to femininity). In Austria, undergraduate men perceive masculinity and femininity as two *different dimensions* and their *estrogen levels are negatively related to masculinity* (but not to femininity).

In the US, undergraduate naturally cycling women perceive masculinity and femininity as two *different dimensions* and their (luteal phase) *estrogen levels are positively related to femininity* (but not to masculinity). In Austria, undergraduate naturally cycling women perceive masculinity and femininity as bipolar ends of one *dimension* and their (follicular phase) *estrogen levels are negatively related to femininity* (and positively to masculinity).

A one-factorial structure seems to correspond to a paradoxically reversed relationship between the "female" sex hormone estrogen and sex role, while a two-factorial structure corresponds to the expected relationship between estrogen and masculinity in men/femininity in women. One psychological mechanism explaining this correspondence could be compensation. If participants assume that being highly feminine makes them less masculine, and vice versa, high estrogen men, who are presumably more feminine, or low estrogen women, who are presumably more masculine, may—in an attempt for socially desirable self-presentation—overcompensate for this fact by rating themselves as more masculine or more feminine, respectively. On the contrary, if participants acknowledge that one can be feminine without having to be less masculine or vice versa, their self-ratings may be more reflective of their actual sex role orientation. However, our findings may also be interpreted in the light of previous research demonstrating that the relationship between sex hormones and behavior is not always linear and may differ between men and women (Tan, 2012).

Culture Reverses Sex Differences in the Factorial Structure of Sex Role Orientation

Furthermore, the sex difference in factorial structure was reversed between the Austrian and US undergraduate samples. In the US sample, men appear to think they can only be feminine when not being masculine, while women incorporate both characteristics. In the Austrian sample however, women appear to think they can only be masculine when not being feminine, while men incorporate both characteristics. These data may reflect a cultural tendency to favor masculine characteristics among US undergraduates as opposed to a cultural tendency to favor feminine characteristics among Austrian undergraduates. This results in a lack of androgynous, but high number of indifferent men in the US sample as opposed to a low number of androgynous, but high number of indifferent women in the Austrian sample. Note however that factorial structures in the Austrian Online sample, which was drawn from a more general population, are more similar to the US data, suggesting a role of education and socioeconomic status in these tendencies. Furthermore, in neither sample did we observe such high numbers of androgynous participants as in Bem's original study (Bem, 1974).

To evaluate, whether these cultural differences may be attributed in culturally different perception of the sex role

concept, US undergraduate and Austrian online participants were asked to explain their sex role ratings. Note that differences in factorial structure were not as extreme between these two samples as between the two undergraduate populations. Nevertheless, curious differences were found between the explanatory variables reported in the two samples. While middle European participants rely most strongly on personality measures to explain their sex role ratings, American participants rely more strongly on activities and interests on the one hand and appearance on the other hand (beauty care). Consequently, while personality is an important dimension of sex role orientation as incorporated in social role theory (Eagly, 2013) and may favor a two-factorial structure, this finding provides support for previous criticism on measures such as the PAQ and BEM, which involve only personality items. Especially, approaches to determine sex role orientation across cultures and seeking to overcome problems with self-reflection and social desirability could profit from including additional dimensions that are more continuous and can be expressed to varying degrees in both men and women.

In summary, we found with respect to our research questions, that neither culture nor menstrual cycle affected sex role ratings *per se*, while OC use did rate themselves more feminine and less masculine than naturally cycling women. However, culture and hormonal status interactively affected whether participants compared themselves more to men or women in their sex role ratings. Also, the impact of sex hormones on sex role orientation was not only modulated by sex and hormonal status, but also by culture. Both culture and hormonal status are also important determinants of the factorial structure of sex role orientation and culture influences which characteristics are viewed as important for sex role orientations by participants themselves. These findings have important implications for the assessment of sex role orientation in different cultures.

Altogether our data are in line with previous psychobiological approaches, suggesting that sex role orientation as well as the sex role self-concept are shaped by both biological mechanisms, such as sex hormones as well as social/cultural influences (Halpern and Tan, 2001).

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