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Review article

A brief guide to the menstrual cycle and oral contraceptive use for researchers in behavioral endocrinology☆

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ABSTRACT

There is increasing evidence that reproductive hormones exert regulatory effects in the central nervous system that can influence behavioral, cognitive, perceptual, affective, and motivational processes. These effects occur in adults and post-pubertal individuals, and can be demonstrated in humans as well as laboratory animals. Large changes in 17β-estradiol and progesterone occur over the ovarian cycle (i.e., the menstrual cycle) and afford a way for researchers to explore the central nervous system (CNS) effects of these hormones under natural physiological conditions. Increasingly, oral contraceptives are also being studied, both as another route to understanding the CNS effects of reproductive hormones and also as pharmacological agents in their own right. This mini-review will summarize the basic physiology of the menstrual cycle and essential facts about oral contraceptives to help novice researchers to use both paradigms effectively.

Over the past 50 years behavioral endocrinology has emerged as a new discipline in the life sciences. Neuroendocrine principles derived from animal studies have also been applied to understanding the *human* brain. These ideas have been especially influential for understanding sex differences in humans, and the sometimes surprising actions of reproductive steroids in the central nervous system (CNS). The earliest human studies, which arose in the mid-1980s, used the concept of organizational effects (Phoenix et al., 1959) to explain behavioral changes found in people with elevated androgen exposure due to congenital adrenal hyperplasia (Resnick et al., 1986) and the concept of activational effects to explain behavioral correlates associated with the human menstrual cycle (Hampson and Kimura, 1988). Mood effects of the menstrual cycle were noted even earlier, but were attributed to other causes (often psychosocial ones, e.g. Blank et al., 1980) because it was not yet understood that adult hormones can affect the function of the CNS by interacting with receptors scattered throughout the cortex and limbic brain regions, including pathways involved in affect and the regulation of emotion (see Toffoletto et al., 2014).

It is now commonplace for researchers to use the menstrual cycle and increasingly, oral contraceptive paradigms, in order to investigate behavioral, cognitive, perceptual, motivational, or affective phenomena associated with changing availabilities of estrogens and progestogens in women. Approximately 500 articles involving the menstrual cycle are published annually (Allen et al., 2016). Excellent studies exist, but there is still considerable misunderstanding, especially by novices but even by seasoned researchers, of the basic physiology or pharmacology of these paradigms. This is an obstacle to using these tools effectively in behavioral endocrine research. On the 50th anniversary of *Hormones and Behavior*, this mini-review will briefly review some basic facts (and common misconceptions) about these two widely used methodologies. More detailed descriptions can be found elsewhere (Becker et al., 2005; Chabbert Buffet et al., 1998; Hampson and Young, 2008).

1. The menstrual cycle

A typical objective in behavioral endocrinology studies is to use the menstrual cycle as a naturalistic method for manipulating the level of the brain's current exposure to 17β-estradiol ("estradiol") and/or progesterone. A CNS outcome of interest is then examined, and usually two or more phases of the menstrual cycle are compared. In contrast to other types of studies, where specific temporal timepoints during the menstrual cycle are the theoretical focus (e.g., studies of premenstrual syndrome) irrespective of the hormone concentrations achieved, here the hormone concentrations themselves are of central importance. Proper timing of the measurements is still essential, though, to maximize the probability that the expected concentration ranges are targeted successfully. The hormone concentrations seen at different stages of the cycle will depend on which phase is targeted, inter- and intra-individual variations in hormone production and release, and key demographic and lifestyle features of the women being studied. Common

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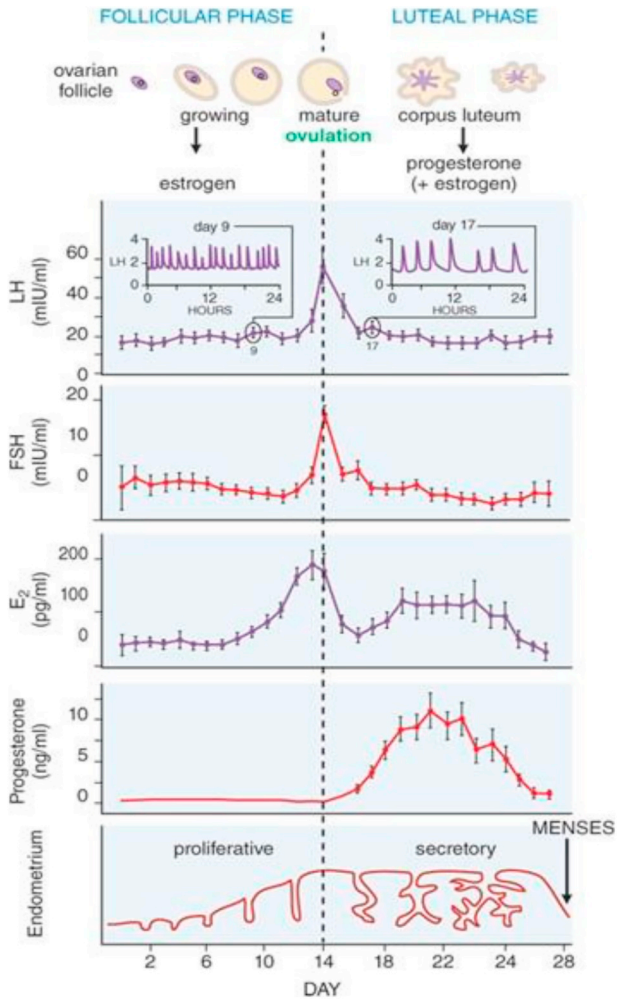

Fig. 1. Typical pattern of changes in: (a) luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E_2), and progesterone, shown over an idealized 28-day menstrual cycle. The dashed vertical line indicates where ovulation occurs. Menstrual bleeding (menses) begins on Day 1 and lasts about 5 days, on average. Mean concentrations ± SE are shown in plasma (Reproduced with permission of McGraw-Hill Education from Levin et al., 2018, *Goodman & Gilman's: The Pharmacological Basis of Therapeutics*, 13th ed (with permission of Copyright Clearance Center Inc) and from Am J Obstet Gynecol, 111, Thorneycroft et al., The relation of serum 17-hydroxyprogesterone and estradiol-17beta levels during the human menstrual cycle, 947-951, 1971 with permission of Elsevier).

study designs used in menstrual cycle research include crossover designs with prospective targeting of pre-defined menstrual cycle phases based on theoretical considerations (e.g., Hampson, 1990a; Maki et al., 2002), or else randomly timed behavioral testing followed by the retrospective 'binning' of women into hormonally homogeneous subgroups (e.g., Hampson and Morley, 2013; Courvoisier et al., 2013). All types of study designs require the retrospective verification of each woman's hormone levels via hormonal assays of serum or saliva (or even urine analysis, if a temporally integrated measure is desirable), and these samples are typically collected during the behavioral testing.

Fig. 1 shows the orderly sequence of hormonal events that unfolds over an idealized ovulatory cycle (see Chabbert Buffet et al., 1998 for a detailed description). A new cycle begins on the first day of menses (menstrual bleeding) which, by convention, is considered Day 1 of the cycle and marks the onset of the follicular (or proliferative) phase (Hall, 2009; see Fig. 1). The first few days are called menses, the menstrual phase, or simply the 'early follicular phase', and represent the phase of the cycle where estradiol reaches its lowest point (about 20–50 pg/mL if measured in standard serum or plasma). On average, menses lasts about 5 days, but can be as long as 7 days. Onset of menses signals that the endometrial lining can no longer be maintained, but estradiol and progesterone may take another couple of days to reach their full nadir (Fig. 1). If it is important for estradiol to be at its lowest point, then testing women in the first two days of the menstrual cycle is best avoided.

Researchers doing behavioral studies frequently assess women at menses because both of the major ovarian steroids, estradiol and progesterone, reach their lowest concentrations making this phase of the cycle a useful baseline point of comparison. The presence of an overt physical marker makes the phase easy to identify. However, the follicular phase as a whole is hormonally heterogeneous; it includes the lowest and highest estradiol levels of the entire cycle. Estradiol is low during menses, but increasing amounts of estradiol are produced by the dominant follicle as it matures over the course of the follicular phase. The mid- to late follicular phase is characterized by rising estradiol, beginning slowly at first, culminating in a high but short-lived peak lasting about 36 h (a range of ~130–200 pg/mL is typical in serum but concentrations as high as 300–400 pg/mL are found in some women). When estradiol reaches a threshold level it triggers a burst in luteinizing hormone (LH) that begins ~24–36 h in advance of ovulation (the release of the mature oocyte by the ovary) (Hall, 2009). Following ovulation, estradiol returns to near menstrual phase values (Abraham, 1978; Fig. 1), only to rise again by production from the corpus luteum during the luteal phase. During the luteal phase estradiol in the bloodstream reaches a more sustained but usually slightly shallower peak in concentration (Chabbert Buffet et al., 1998; Levin et al., 2018). The marked drop in estradiol that precedes the luteal rise is not always readily visible in diagrams like Fig. 1, if averaged instead of individual concentrations are plotted by day of cycle. This is because the exact timing of ovulation varies, attenuating the dip that's evident in averaged data.

Following ovulation, the residual cells left by the ovulatory follicle are called the corpus luteum. It serves as a source of progesterone, estradiol, and inhibin A in the last 2 weeks of the cycle (Chabbert Buffet et al., 1998). Ovulation, and the formation of the corpus luteum, marks the beginning of the luteal (or secretory) phase of the cycle, which is conventionally thought to be relatively fixed in length at ~13–15 days (Vollman, 1977; but see Bull et al., 2019), though in certain women (notably older ones) it may be as short as 10 days (Hall, 2009). During the luteal phase, estradiol and progesterone both rise to a sustained peak, which is usually evident during the interval from 10 days to 5 days before onset of the next menses (i.e., during the 'midluteal' phase), and lasts about 5–6 days. Plateau levels of estradiol are about 100–150 pg/mL (Abraham, 1978; Thorneycroft et al., 1971). Production of progesterone by the ovaries is negligible until the luteal phase begins (i.e., until the corpus luteum forms). Therefore serum concentrations of progesterone are low and nearly undetectable during the follicular phase of the cycle. However, a very small increase in progesterone just prior to ovulation can be seen and is thought to assist in bringing about follicular rupture (Hall, 2009).

During the peak in luteal production, the progesterone concentration is ~10–20 ng/mL (Abraham, 1978; Thorneycroft et al., 1971) and tends to coincide temporally with the sustained peak in estradiol usually evident during the middle of the luteal phase. In some cycles, however, the peak in hormone output may occur slightly earlier or later during the luteal phase (Beltz and Moser, 2019), making a midluteal maximum a reasonable but sometimes inaccurate assumption. One difficulty for researchers is that there is no point in the menstrual cycle where high (or low) progesterone can be assessed on its own, independently of high estradiol. As the luteal phase draws to a close, the corpus luteum begins to regress, and its production of estradiol and progesterone falls. This is sometimes called the premenstrual phase of the cycle and lasts about 3–4 days. Although both hormones decline, they do not always fall in synchrony at the close of the luteal phase

(Hampson and Young, 2008). Once the endometrium can no longer be sustained and begins to shed it marks the beginning of menses and the onset of a new menstrual cycle.

This seemingly straightforward sequence of events contains many potential pitfalls for the unwary researcher. Although the sequence is fixed, assuming a cycle is ovulatory, the exact timing of the individual events and levels of hormones achieved at each stage varies significantly from woman to woman and cycle to cycle. If it is important in a given study for behavior to be tested under particular hormonal conditions (e.g., when estradiol is high, but progesterone is still negligible), then it is essential to measure the target hormones in serum or saliva to assure those conditions were successfully met (see Hampson and Young, 2008; Hofman, 2001; Schultheiss et al., 2018, for a discussion of assay methods). Accurate timing of the behavioral testing (e.g., if the desired target is 6–7 days before the onset of the next menstrual period, and this timing is in fact verified by a retrospective day count, as described in Hampson and Young, 2008) is not by itself a guarantee, although it does increase the probability that the expected concentration ranges will be verified by objective assay methods. Because the presence of menses can only occur physiologically if circulating levels of ovarian steroids are extremely low, low concentrations of estradiol and progesterone can be safely assumed if menses is actively in progress. Even then, however, assays are still desirable if there is any uncertainty about the accuracy of women's self-reports.

It is important for researchers to ascertain the usual length of each individual woman's cycle. A 28-day cycle is 'classic' but found in only about 15% of women (e.g., Bull et al., 2019). Thus a 28-day cycle cannot be assumed. Large-scale studies show the average cycle is about 29.5 days, but varies considerably across individual women; normal ovulatory cycles can be as short as 24 days or as long as 35 days (Treloar et al., 1967). (Shorter or longer cycles, outside of the 24–35 day window, may occur in any sample of women but are significantly less likely to be ovulatory). Nor is it appropriate for a researcher to 'standardize' cycles to a 28-day standard, because this falsely assumes that other cycle lengths can simply be stretched or shrunken to fit a 28-day frame. In fact, however, most of the variation across women in cycle length is due to variations in the length of the follicular not luteal phase (Levin et al., 2018). The luteal phase remains relatively fixed at 13–15 days irrespective of the overall cycle length (Vollman, 1977; but see Bull et al., 2019), although a foreshortened luteal phase can sometimes be seen, e.g., among older women in their 40s or in women who are very athletic (Burrows and Bird, 2000). This asymmetry in the follicular and luteal phases also means that the timing of ovulation will not be at 'mid-cycle' in a great many women. A woman with a 35-day cycle will ovulate on ~Day 21, for example, whereas a woman who has a 24-day cycle will ovulate on about Day 10. Timing of research sessions may need to be adjusted accordingly (Hampson and Young, 2008 for further discussion).

To further complicate the life of researchers, there are *intra*-individual differences in the length of the cycle as well as *inter*-individual differences. Women vary in how predictable their cycles are: some have cycles that can be predicted with great accuracy. Typically, however, the onset of menses varies from one cycle to another by 2–4 days. Data based on more than 25,000 person-years of menstrual history showed that 75% of all cycles among women ages 20–40 vary by less than 6 days (Treloar et al., 1967). This is normal range variation; if a woman does not ovulate in a particular cycle, departure from her average cycle length may be considerably larger. To establish average cycle length, verbal report is of questionable validity (Bean et al., 1979). Some women methodically keep track of their menstrual cycles and can provide accurate information, but for many others, who either do not keep track (or who misunderstand how to properly do day counts), informal estimates of cycle length and variability can depart substantially from estimates that are based on objective measures. If in doubt, a researcher may need to track several ovarian cycles in advance of having a woman participate in research, to assess true cycle length through objective record-keeping. This can be accomplished through methods such as menstrual diaries, digital apps (e.g., Clue, Period Tracker), or simply recording the exact date of onset of menses over several consecutive cycles.

Aside from individual differences in the *timing* of the hormonal events, the *amplitudes* of those same hormonal events also vary widely. This reflects individual differences in the genetic machinery responsible for steroid production and metabolism, but also environmental influences, including diet (Ellison et al., 1989; Pirke et al., 1985), physical or psychological stressors including acute illnesses (Breen et al., 2004; Sutter and Schwartz, 1985), levels of exercise (Ellison and Lager, 1985; Kasa-Vubu et al., 2004), or other factors beyond the scope of the present mini-review (see Hampson and Young, 2008). Perhaps the single most important determinant of the levels of hormones observed is a woman's chronological or reproductive age, which has an impact on the regularity of the menstrual cycle, the probability of anovulatory cycles, and the concentrations of ovarian hormones that are observed. Ovarian function peaks later and starts to decline earlier than what is implied by the presence of menstrual regularity alone. Full ovulatory and luteal phase sufficiency is evident between ages mid-20s to about age 35 (Lipson and Ellison, 1992). The average levels of ovarian hormones at age 18–19 are only ~50% of those of women in their mid-20s or older (Asso, 1983; Read et al., 1984). If hormone levels matter, it may be relevant to consider whether very young women have sufficient levels of hormones to affect the phenomena under study. The advent of menstrual cycling at menarche does not imply that full ovulatory competency has been attained. Cycles in the first few years after menarche is achieved often tend to be irregular and exhibit a high incidence of anovulatory cycles. By 5 years post-menarche, the frequency of anovulatory cycles decreases to ~25%. In contrast, full reproductive maturity in women is associated with a very high proportion of ovulatory cycles. Metcalf and Mackenzie (1980) studied ovulation in 254 women over 3 months. Ovulation took place in every cycle in 62% of women aged 20–24 years, in 88% of women aged 25–29 years, and 91% of women over 30. For research where full ovulatory competence is important, women from their mid-20's to mid-30's are the ideal subjects.

Once full reproductive maturity is achieved the menstrual cycle remains fairly constant but shortens gradually with aging until roughly gynecologic age 25 years (i.e. 25 years after menarche) (e.g., Bull et al., 2019). Shortening reflects mostly a decrease in the follicular phase (Sherman et al., 1976; Hall, 2009), although some luteal phase shortening may also be present (Santoro et al., 1996) albeit to a lesser degree. The term "perimenopause" is sometimes used to describe the menopausal transition in the 2 to 8 years leading up to menopause. In the early part of the transition, menstrual cycles are regular and women may appear to have normal cycles, but anovulatory cycles become more frequent. In the later transition, the length of the cycle becomes increasingly irregular and menstrual periods may be missed (Vollman, 1977). It was formerly believed that estradiol production declined progressively throughout perimenopause, but some data suggest that in the early stages of the transition elevated FSH can lead to *increased* estradiol production relative to women under age 35 (e.g., Santoro et al., 1996; Burger, 1996). Eventually, estradiol levels do decrease but this is a late transitional event. There may also be changes in expression of target tissue estradiol receptors during the perimenopause, though this is not well-documented (Prior, 2005).

To do menstrual cycle research effectively, researchers often need to evaluate whether participants have medical conditions or are taking any drugs that might alter either the temporal patterning or endocrine profile of the menstrual cycle (Hampson and Young, 2008). Medications that alter characteristics of the menstrual cycle include antipsychotics, psychotropic drugs used as mood stabilizers, and anticonvulsants. Even routine antibiotics can produce changes in steroid metabolism (Stanczyk et al., 2013). Oral contraceptives (OCs) are the class of medications that have the largest effects on a woman's

menstrual cycle. Recently, OCs have become of interest to behavioral endocrinologists in their own right (e.g., Montoya and Bos, 2017; Cahill, 2018; Pletzer and Kerschbaum, 2014). Accordingly, we end this mini-review by briefly highlighting the major features of oral contraceptives.

2. Oral contraceptives

A majority of North American women use oral contraceptives at some point during their reproductive years (Daniels et al., 2013). Although predominately prescribed for contraception, OCs may also be prescribed for other reasons (De Leo et al., 2016) and can be taken on either a short-term or long-term basis. Most OCs sold in the U.S. and Canada are "combined" OCs, which contain a synthetic estrogen (ethinyl estradiol) in combination with one of at least 12 different progestins (synthetic forms of the naturally-occurring hormone progesterone). Mestranol, another estrogen formerly used in OCs, is rarely used in OCs today. The different progestins used in OCs vary in progestational potency and, in addition to their primary role as progestins, exert estrogenic, anti-estrogenic, androgenic, or anti-androgenic activity in varying degrees (De Leo et al., 2016). The progestins contained in OCs are often classified into 4 or 5 'generations', reflecting salient differences in their pharmacological properties (Petitti, 2003). For example, the progestin drospirenone is based on a different molecular backbone and has anti-androgenic rather than androgenic effects in tissues (Fuhrmann et al., 1996).

The daily estrogen dosage contained in OCs varies by brand. Because brands also vary in their progestin dosage and the profile of endocrine effects associated with the specific progestins they contain, it is important to understand that not all OCs are alike. In research studies where the physiological effects of OCs are relevant, it is usually undesirable to lump heterogeneous OC users together as a single group. Doing so can obscure potentially important diversity among the different OC formulations. As one concrete example, increasing evidence suggests that women's performance on certain cognitive visuospatial tasks (e.g., mental rotation) is modestly sharpened by some types of OCs but hampered by others (e.g., Wharton et al., 2008; Griksiene et al., 2018). If a researcher's intent is to study the effects of OCs on CNS variables, it is important to identify the exact type of OCs being studied.

OC medications have profound effects on the menstrual cycle. As a primary mechanism, OCs prevent ovulation through negative feedback inhibition at the hypothalamus (Elliott-Sale et al., 2013; Levin et al., 2018). The use of OCs by women is associated with a marked reduction in the endogenous production of both 17β-estradiol and progesterone. Circulating concentrations of these two steroids during periods of active pill use approximate menstrual phase values, where hormone output by the ovaries is minimal (Elliott-Sale et al., 2013). Production of testosterone by the ovaries and adrenals is also greatly decreased (e.g., Zimmerman et al., 2014). OC usage is associated with *increases* in the levels of circulating SHBG (e.g., Panzer et al., 2006; Stegeman et al., 2013) owing to increased hepatic production driven by the exogenous hormone intake, which further reduces the testosterone that is available to the body biologically (Zimmerman et al., 2014). Bioavailable levels of testosterone in OC users are reduced by ~50–60% (Snihur and Hampson, 2012; Wiegratz et al., 2003; Zimmerman et al., 2014). Ethinyl estradiol, the synthetic estrogen contained in OCs, has low affinity for SHBG (Stegeman et al., 2013; Stanczyk et al., 2013) and thus in the circulation it is largely bound to albumins (97–98%) and not SHBG (Kuhnz et al., 1990; Levin et al., 2018; Orme et al., 1989).

While endogenous hormones are inhibited, the exogenous hormones supplied by OCs do exert biological activity. In the 1960s when OCs were first introduced, they contained a high estrogen content (up to ~150 μg) and 1 to 10 mg of progestin. These OCs were associated with undesirable side effects on women's physiology (e.g., increased risk of hypertension, thrombosis, adverse effects on glucose metabolism, Stanczyk et al., 2013). This gave rise to the idea that OC use produces a high, perhaps supraphysiological, estrogen state. However, the dosage of hormone contained in OC pills has been progressively reduced by the manufacturers over the last few decades, while preserving their contraceptive efficacy. Presently, OC pills typically contain only 15 to 35 μg of ethinyl estradiol and a commensurately lowered progestin content. Although available serum concentrations of the exogenous hormones are of course higher during weeks of active pill use than during the 'inactive' week of each contraceptive cycle, it is by no means clear that they exceed normal physiological levels of exposure. One indicator of the relatively low hormone content of today's OCs are reports of decreased accumulated bone density, relative to girls not taking OCs, in girls using OCs over a sustained period during their teen to young adult years when peak bone deposition is normally occurring with the support of estradiol (Teegarden et al., 2005; Cromer et al., 2004).

Most brands of OCs involve a recurring contraceptive cycle of 21 consecutive days of OC pill use followed by a pill-free week (or a week of inert placebos containing no hormone (Levin et al., 2018). Menses occurs during the 'inactive' week. Alternative regimens with a 24- or even 84-day active phase have been developed, but have been adopted by only small numbers of women. Some OC brands have a fixed daily dosage (monophasic pills), but in bi- or triphasic brands ('multiphasics') dosages of the estrogen and/or progestin constituents are adjusted over the 21-day contraceptive cycle to better imitate the changes in ovarian hormones that occur over a natural menstrual cycle (De Leo et al., 2016). (The hormonal conditions generated by biphasic or triphasic OCs still differ significantly from a non-OC cycle, however, otherwise their contraceptive efficacy would be lost). Although contraceptive steroids differ in their molecular structure from the naturally-occurring forms of the hormone, ethinyl estradiol binds to estrogen receptors (or at least, ERα) with affinity similar to, or perhaps even greater than, 17β-estradiol itself (Briggs and Briggs, 1983; Blair et al., 2000). Barkhem et al. (1998) reported that ethinyl estradiol showed preferential activity at ERα over ERβ, and thus may alter the balance between alpha and beta signaling compared to the endogenous ligand, 17β-estradiol (see also Raval et al., 2012; Escande et al., 2006). For researchers interested in the CNS, it is important to know that ethinyl estradiol does, in fact, enter the CNS (Fishman and Norton, 1977) and thus in principle is capable of influencing CNS function.

One relevant consideration for researchers studying OCs is the time course of the drug; peak concentrations in the bloodstream are typically seen 1.5–2 h after oral ingestion, followed by a fairly rapid return to basal values via tissue deposition and, mainly, metabolic deactivation (DiLiberti et al., 2011; see Fig. 2). One unresolved difficulty facing researchers is that the time course by which any CNS effects appear in response to pill ingestion is currently unknown–and if genomically-mediated these CNS effects need not coincide with the peak concentrations of the contraceptive steroids evident in serum or plasma. Recent work suggests that the timing of behavioral measures may matter (Peragine et al., 2019), and in recognition of this uncertainty recorded whether their behavioral testing took place soon after, or more distal to, the timing of each woman's daily pill ingestion.

Yet another practical problem facing researchers is how to quantify the effective level of hormone exposure in women taking OCs. Tables are available that rank the relative estrogenic and progestational potencies of various OC brands based on bioassays (e.g., their effects on the mouse endometrium). However, individual differences in steroid absorption and metabolism might be relevant to differences in behavioral outcomes that are seen across individual women. The exact extent to which metabolic differences actually occur, however, is controversial. Early work suggested that even if the daily dosage administered is fixed, there is substantial variation from woman to woman in the resulting blood levels of ethinyl estradiol and progestin achieved while taking any particular OC formulation (e.g., Goldzieher and Stanczyk, 2008). But recent papers argue that in fact there is much less intersubject variability in the pharmacokinetics than those earlier

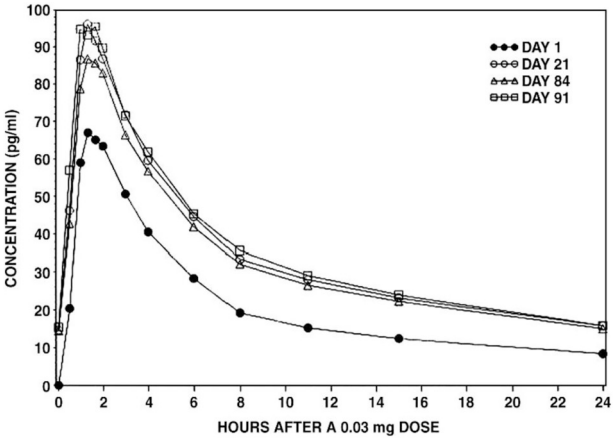

Fig. 2. Mean concentrations of ethinyl estradiol measured in 30 healthy female volunteers on Days 1, 21, 84 and 91 of a contraceptive cycle in which 30 μg ethinyl estradiol was administered daily in combination with 0.15 mg levonorgestrel. Blood was taken at 12 time points post dose. Plasma was analyzed for ethinyl estradiol using a liquid chromatography-tandem mass spectrometry method. Note the rise to peak values within 2 h post ingestion, followed by rapid clearance of the steroid from the bloodstream. Peak concentrations are lower on Day 1 than later in the cycle, after steady-state concentrations are reached under repeated dosing. (Reprinted from DiLiberti et al., 2011, *Contraception*, 83, Steady-state pharmacokinetics of an extended-regimen oral contraceptive with continuous estrogen, pp. 55–61, with permission from Elsevier).

papers implied (Jusko, 2017), and that while the levels of OC steroids attained do vary, they only do so within fairly narrow ranges. A recent meta-analysis by Jusko (2017) of 6 different OC products containing 30 μg of ethinyl estradiol and 150 μg of levonorgestrel tested across 17 different empirical studies found that on average the peak concentration of ethinyl estradiol seen in plasma sampled 1.5–2 h after pill ingestion was consistently about 99 pg/mL for all 6 formulations, with a subsequent trough level of 18 pg/mL preceding the next OC pill administration. Variation around these means was quite low. Peak concentrations reached after a 20 μg dose of ethinyl estradiol are correspondingly lower (Boyd et al., 2003). These values reflect the steady-state concentrations achieved after several days of OC pill use; a substantially lower peak can be observed at the beginning of a contraceptive cycle (DiLiberti et al., 2011; see Fig. 2). It can take several days for steady-state concentrations to become established.

At first glance, an easy solution to assess individual differences is to use commercially sold immunoassay techniques (e.g., enzyme immunoassay, radioimmunoassay) to quantify the serum (or salivary) concentrations of the exogenous steroids attained in each woman. Because of differences in their molecular structure, however, contraceptive steroids typically show very limited cross-reactivity with the antibodies used in these standard assays, which are designed to measure the naturally-occurring forms of estradiol or progesterone with very high specificity. Most commercially available assays show cross-reactivities of well under 1%. This tells us that measuring hormones in OC users by employing the standard assays used routinely in hospitals or clinical laboratories, no matter how excellent those assays may be, is not an accurate or effective method to quantify the concentrations of the exogenous OC steroids that are present. In contrast, measuring *endogenous* steroids in women using OCs *can* be done using those same methods and typically reveals serum concentrations as low or lower than those seen at the menstrual phase of the cycle in non-OC users (e.g., De Leo et al., 1991; Elliott-Sale et al., 2013). This reflects the inhibitory effects of OCs on endogenous ovarian production. Unfortunately, the endogenous hormone complement does not represent the total hormone complement that is actually present. Antibody with high specificity for ethinyl estradiol does exist but is not widely available outside of pharmaceutical settings (e.g., Dibbelt et al., 1991).

OCs are the most widely used method of hormonal contraception, but other hormonal methods are also available (e.g., depot medroxyprogesterone acetate). Any hormone-based contraceptive will affect the endocrine milieu. Thus it is incumbent upon researchers studying the menstrual cycle to inquire what type of contraception their study participants are using and to determine what effects, if any, those contraceptives might have on women's endocrine profiles.

3. Conclusions

Over the past 50 years, the lifetime of the journal *Hormones and Behavior*, menstrual cycle studies and oral contraceptive studies have begun to be used as paradigms to investigate the effects of reproductive steroids on CNS function under 'real world' conditions. These are important paradigms for researchers studying young women where treatment with exogenous estrogens in the absence of clinical indications is not usually permissible. Early studies applied these methods to cognitive functions that show sex differences with a suspected hormonal origin (Hampson, 1990a, 1990b) or to mood changes associated with the menstrual cycle (Halbreich et al., 1986; Bäckström et al., 2003), but in recent years menstrual cycle paradigms have been applied to an expanded range of phenomena (e.g., DeBruine et al., 2010; Jünger et al., 2018; Lobmaier et al., 2015). Although there are still relatively few studies, menstrual cycle paradigms are now being used in conjunction with functional brain imaging techniques to visualize menstrual cycle-dependent changes in the activation of specific brain regions or larger brain networks (Sacher et al., 2013; Jacobs and D'Esposito, 2011). For example, work is ongoing on the modulation of the reward system by gonadal steroids (Ossewaarde et al., 2011). Imaging studies are especially ripe for future investigations; their findings are relevant not only to neuroendocrinologists who study hormones but also to general neuroscientists who do ordinary functional imaging work and are still unaware that changes in the endocrine environment can affect the patterns and magnitudes of brain activations observed with modern imaging techniques (see Cahill, 2012).

With regard to OCs, early studies that suggested that OCs might potentially affect brain function (e.g., Hampson, 1990c) were generally overlooked or viewed with skepticism because of the widespread and entrenched idea that OCs did not affect the CNS. Increasingly there are reasons to question that entrenched wisdom, and over the past 5 years a number of researchers have called for further studies to investigate whether and how OCs might affect the brain (e.g., Montoya & Bos, 2017; Pletzer and Kerschbaum, 2014). OCs have been shown to alter how certain types of memories are coded (Nielsen et al., 2011), and to alter patterns of neural activity among women currently using OCs (Pletzer et al., 2014; Petersen et al., 2014). Certain abilities such as mental rotation may be modestly influenced by OC usage (e.g., Peragine et al., 2019), with the direction and size of the effect depending on the androgenic activity of the progestins contained in OC formulations (Wharton et al., 2008; Griksiene et al., 2018), and ethinyl estradiol dosage (Beltz et al., 2015). OC usage has been linked to changes in women's sexual attractiveness and conversely the mates they find attractive (Birnbaum et al., 2019; Welling, 2013), changes in emotional responsivity (Hamstra et al., 2014; Montoya and Bos, 2017), reduced sensitivity to certain odours (Renfro and Hoffmann, 2013), and changes in libido (Zethraeus et al., 2016). Many of these phenomena, if replicated, will have implications for daily life and especially the social relationships of women currently using OCs. Future research will need to carry these results forward with a clear and well-informed knowledge of OC physiology. Early studies (and even some recent ones) often suffered in quality methodologically from an inadequate understanding of how OCs affect women's bodies. Increasing evidence of CNS-related effects raises many important theoretical questions, such as whether any OC effects on the brain will reverse once the OCs are discontinued (or conversely, whether there are lingering effects, Egan and Gleason,

2012), and whether the use of OCs at puberty (often suspected to be an 'organizational' period when steroid exposure may be able to bring about lasting changes in the CNS; Sisk, 2016) might be associated with enduring changes in the CNS as a result of exposure to the exogenous steroids (Cahill, 2018). It will be exciting to watch the next 50 years unfold.

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Appendix A. Tips for researchers

When studying the menstrual cycle:

- Relevant hormones should be measured in serum or saliva in order to verify that concentrations meet study criteria. Invalid datapoints should be removed. For example, if the study requires testing women who have ovulated, then women whose luteal phase hormones prove to be inconsistent with ovulation should not be considered, as they are an invalid test of the hypothesis.
- Because hormone levels may overlap at different phases of the menstrual cycle, recording individual day-of-cycle information (i.e., the day of each woman's cycle when the samples were collected) can help to identify the correct menstrual cycle phase if hormone levels are ambiguous. However, day-of-cycle information is not sufficient on its own whenever hormone levels are of interest.
- Peak estradiol concentrations have already occurred by the time the LH peak is seen at ovulation. LH monitoring is therefore not particularly effective for prospectively identifying the pre-ovulatory estradiol surge, but it can help to identify the fertile window when conception can take place.
- Full adult levels of ovarian hormone secretion are not attained until women are in their early 20's, on average. This may be a relevant consideration for deciding what ages to study.
- A 28-day cycle cannot be assumed. Normal ovulatory cycles range from about 24–35 days in length.
- A rise in basal body temperature after ovulation is not always observed, even if a woman has in fact ovulated (Moghissi, 1976).
- Many women are inaccurate at reporting the average length and variability of their menstrual cycle, and can't reliably identify the exact date of onset of their last menstrual period unless they formally keep track of it through calendar methods or digital trackers. Treat self-reports as questionable.
- A reverse day count beginning at Day 1 of the cycle and counting backwards into the previous luteal phase has moderate-to-high accuracy in identifying the luteal phase because the length of the luteal phase is relatively fixed and predictable. However, day count alone cannot confirm or disconfirm whether ovulation successfully occurred.

When studying oral contraceptive effects:

- Researchers should typically report which type(s) of OCs the women in their sample were using.
- The CNS effects of OCs may depend not just on dosages, but also on the specific progestins that are used. The various progestins contained in OCs have differing endocrine profiles.
- For typical 'combination' OCs, there is no point in the contraceptive cycle where the estrogen and progestin components of the pills are separated from one another. Combination OCs thus do not allow the effects of the two steroids to be assessed independently.
- Standard immunoassays are typically ineffective for measuring contraceptive steroids.
- Researchers must be careful not to overgeneralize study findings: it is seldom appropriate to generalize to OCs in general, particularly if only a single brand of contraceptive has been studied.
- Similarly, one should not generalize findings that are based on oral contraceptives to hormonal contraceptives more generally, unless a broader range of hormonal contraceptives has actually been studied.

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