0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(4):1650–1673

doi: 10.1210/en.2004-1142

Strategies and Methods for Research on Sex Differences in Brain and Behavior

Jill B. Becker, Arthur P. Arnold, Karen J. Berkley, Jeffrey D. Blaustein, Lisa A. Eckel, Elizabeth Hampson, James P. Herman, Sherry Marts, Wolfgang Sadee, Meir Steiner, Jane Taylor, and Elizabeth Young

Department of Psychology (J.B.B.), University of Michigan, and Department of Psychiatry (E.Y.), University of Michigan Medical School, Ann Arbor, Michigan 48109; Department of Physiological Science (A.P.A.), University of California at Los Angeles, Los Angeles, California 90095; Program in Neuroscience (K.J.B.) and Department of Psychology (L.A.E.), Florida State University, Tallahassee, Florida 32306; Center for Neuroendocrine Studies (J.D.B.), University of Massachusetts, Amherst, Massachusetts 01003; Department of Psychology (E.H.), University of Western Ontario, London, Ontario, Canada N6A SC2; Department of Psychiatry (J.P.H.), University of Cincinnati, Cincinnati, Ohio 45237; Society for Women's Health Research (S.M.), Washington, D.C. 20036; Department of Pharmacology (W.S.), Ohio State University, Columbus, Ohio 43210; Department of Psychiatry and Behavioral Neurosciences (M.S.), McMaster University, Hamilton, Ontario, Canada L8N 4A6 and Department of Psychiatry (J.T.), Yale University School of Medicine, New Haven, Connecticut 06520

Female and male brains differ. Differences begin early during development due to a combination of genetic and hormonal events and continue throughout the lifespan of an individual. Although researchers from a myriad of disciplines are beginning to appreciate the importance of considering sex differences in the design and interpretation of their studies, this is an area that is full of potential pitfalls. A female's reproductive status and ovarian cycle have to be taken into account when studying sex differences in health and disease susceptibility, in the pharmacological effects of drugs, and in the study of brain and behavior. To investigate sex differences in brain and behavior there is a logical series of questions that should be answered in a comprehensive investigation of any trait. First, it is important to determine that there is a sex difference in the trait in intact males and females, taking into consideration the reproductive cycle of the female. Then, one must consider whether the sex difference is attributable to the actions of gonadal steroids at the time of testing and/or is sexually differentiated permanently by the action of gonadal steroids during development. To answer these questions requires knowledge of how to assess and/or manipulate the hormonal condition of the subjects in the experiment appropriately. This article describes methods and procedures to assist scientists new to the field in designing and conducting experiments to investigate sex differences in research involving both laboratory animals and humans. (*Endocrinology* 146: 1650–1673, 2005)

WITHIN THE BASIC AND CLINICAL scientific community, there is increasing recognition that differences between males and females, across the lifespan, affect an individual's health, his/her development of disease, signs and symptoms of pathophysiology, and response to therapy. Scientists new to the field, however, can be daunted by methodological concerns, which represent a serious barrier to research. Potential investigators of sex/gender difference want to be know the answers to the following questions. What is a good experimental design for studying sex/gender differences in humans and other animals? What controls do we need? How do we consider hormones? How do we best measure or otherwise assess and manipulate hormonal milieu? How do we incorporate interacting sex/gender factors in our experimental design and in interpreting our data? How will studies that include or add assessments of sex/gender issues affect costs? Although investigators who are new to the field can find some answers by scouring the literature or consulting experts who do such research, there are no published sources currently available that provide overall and comprehensive guidelines.

This article was conceived from discussions held among the members of the Isis Fund Network on Sex, Gender, Drugs and the Brain, sponsored by the Society for Women's Health Research. The Society for Women's Health Research is dedicated to building research capacity to study biological differences between males and females. Our discussions lead us to conclude that the difficulty of working with both male and female animals to scientists not familiar with all of the considerations and pitfalls is a major barrier to advances in the field. The purpose of this monograph is to offer guidelines and, in doing so, facilitate the initiation of new research. The intention is to give investigators interested in pursuing research on sex differences as many resources as possible to enable development and data interpretation of a well-designed study. It is important to note, however, that this monograph should be viewed simply as a guide, not a prescription for the right way to do such work.

The article begins with an overview of mammalian sexual differentiation and ontogeny of sex differences. Then it answers questions that new investigators might pose with ad-

First Published Online December 23, 2004

Abbreviations: CAH, Congenital adrenal hyperplasia; HPA, hypothalamic-pituitary-adrenal; HPG, hypothalamic-pituitary-gonadal; HRT, hormone replacement therapy.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

ditional references for more details. Because many of the questions are appropriate for both clinical and basic research, this manuscript provides guidance for both.

Introduction

Sexual dimorphism in mammals begins early during development as a result of a combination of genetic and hormonal events and continues throughout the lifespan. Physiological sex differences account for marked differences in disease incidence, manifestation, prognosis, and treatment observed between the sexes. Differences in disease susceptibility, time of onset of symptoms, and drug response have become evident in diseases such as cancer, obesity, coronary heart disease, and autoimmune and mental health disorders, among others. In 2001, the Institute of Medicine (IOM) Committee on Understanding the Biology of Sex and Gender Differences published its findings in a landmark report, "Exploring the Biological Contributions to Human Health: Does Sex Matter?" (1). This report highlights the need to conduct hypothesis-driven biomedical studies that take into account sex as a basic human variable. The IOM report validates the need for research on sex-based differences at the molecular, cellular, and whole organism levels and at different stages of the life span. The IOM committee further recommends the use of animal models that best mimic conditions being addressed.

Although researchers from a myriad of disciplines are beginning to appreciate the importance of considering sex differences in the design and interpretation of their studies, this is an area that is full of potential pitfalls. A female's reproductive status and ovarian cycle have to be taken into account when studying sex differences in health and disease susceptibility or in the pharmacological effects of drugs. To facilitate the consideration of these variables in work in different fields, it is our goal to discuss many of the issues that are important for proper design of studies on sex differences.

Definition of terms (see also the glossary at the end of this article)

A sex difference in a trait occurs when normal males and females of a species differ in that trait. The trait is said to be sexually dimorphic because it occurs in two forms, one form typical of males and the other typical of females. Traits may differ in their mean, variability, temporal progression, or developmental profile. Sex differences may be transient (reversible) or permanent.

Masculine and feminine are used to describe traits that are typical of males and females of a species, respectively.

Masculinization and feminization refer to any change that makes an animal more like typical males or females.

Demasculinization and defeminization refer to any change that makes an animal less like typical males or females.

Masculinization and defeminization are not synonymous because the differentiation of tissues subserving feminine functions can be independent of differentiation of tissues subserving masculine functions (2). For example, some treatments may masculinize, but not defeminize.

Although these definitions are rather general, it should be noted that in the behavioral neuroendocrine literature, masculinization and feminization typically refer to permanent (organizational) effects of testosterone and its metabolites, usually not to the more transient (activational) effects of hormones in adulthood. The present review emphasizes work on mammals, but many of the conceptual issues and experimental designs can be applied to other species, especially vertebrates.

Conceptual framework: the origins of sex differences

All biological sex differences are initiated by genes encoded on the sex chromosomes. All other known inherited factors (*e.g.* autosomal genes, mitochondrial genes, and cytoplasmic factors derived from the egg) on average are thought to be inherited equally by males and females. Although two individuals may differ in any of these inherited factors, the only factors that are consistently inherited in a sex-specific fashion are those on the sex chromosomes. The difference between XY and XX cells is potentially attributed to 1) the presence of Y genes only in male cells, 2) the potentially higher dose of X genes in XX cells than in XY cells; and 3) the presence of a paternal genomic imprint on the X chromosome that occurs only in females (see Ref. 3 for further discussion).

A critical gene on the mammalian Y chromosome is *SRY*, the testis-determining gene. This gene causes the embryonic undifferentiated gonad to develop into a testis rather than an ovary (4). This developmental decision is the primary event determining whether an individual is phenotypically male or female. The embryonic testes secrete three hormones: testosterone, Müllerian-inhibiting hormone, and IGF-3. Müllerian-inhibiting hormone causes regression of the Müllerian ducts, which are otherwise destined to form the oviducts and upper vagina. IGF-3 is required for descent of the testes. These hormones act on specific cells throughout the body and brain and cause them to develop in a masculine (male-typical) fashion (5, 6). For example, testosterone enters the brain during early critical periods of development and causes the formation of neuronal cell groups and synaptic connections that control functional and behavioral traits that are more common in males than in females. In short, testosterone (and depending on the species, its metabolites dihydrotestosterone and/or 17β-estradiol) masculinizes the brain permanently by acting during early critical periods of neuronal development (7–10). When testosterone concentrations are low, as occurs typically in genetic females, the development of the brain is feminine. Testosterone also acts elsewhere in the body to cause masculine patterns of development. It acts on the urogenital primordia to cause formation of a penis rather than clitoris, and a scrotum rather than vaginal labia. Thus, the initial commitment of the primitive gonad to a testicular fate results in widespread hormone-driven masculinization of numerous tissues.

The actions of *SRY* and testosterone also have an indirect effect on the environments that a young animal or person will experience. In human societies, the sex of a baby is usually judged by the genital morphology at birth, the presence of a penis or vagina. Once the sex is determined, the baby or young child is treated differently depending on whether it is a boy or girl. In rodents, the mother licks the perineal region

of her sons more than those of her daughters (11–13). These differences in environment have profound effects on the development of gender-specific behavioral repertoires. The sexual differentiation of the body also means that males and females have different bodily sensations (*e.g.* those arising from male *vs.* female reproductive functions) and thus can be conditioned differently by those experiences. Although the effects of *SRY* and testosterone on formation of the urogenital system are events that happen prenatally in humans and other mammals, the effects of hormones on the developing central nervous system extend into early postnatal life in many species, including rats and mice.

Long after the fetal and postnatal periods of sexual differentiation of the brain and body, the gonads continue to be a major contributor to sex differences in brain and behavioral traits. In laboratory rodents, for example, males are exposed to high concentrations of testosterone, which is secreted by the testes more or less continuously. In contrast, the major secretions of the ovary are estrogens (particularly the potent estrogen, 17β-estradiol) and progestins (particularly progesterone), which fluctuate in concentration during the estrous cycle and pregnancy. These circulating gonadal steroid hormones act to influence brain activity differently in males and females. (Large fluctuations in hormone concentrations occur in both sexes in seasonally breeding mammals, because of the period of reproductive quiescence tied to specific environmental conditions.) Traditionally these activational effects are transient (they do not last long after steroid concentrations decline) and are contrasted with the permanent organizing or differentiating effects of early gonadal secretions (14, 15).

Virtually all sex differences in brain and behavioral phenotypes are attributed to the actions of gonadal steroids, in combination with environmental events, on the brain and other target tissues. The genetic sex of brain cells is also theoretically a source of sexually dimorphic information. For example, genes encoded on the Y chromosome are expressed in the brains of males and could have a male-specific effect if other genes in females do not mimic their role. Moreover, genes encoded on the X chromosome may be expressed at a different level in the brains of females and males. In a few cases, these putative sex-specific, cell-autonomous actions of sex chromosome genes have been implicated as the cause of some sex differences in the brain and other tissues in mammals and birds (16). Beyond the roles of gonadal steroids and genes, behavioral phenotypes in humans may also be influenced by sex differences in experience or cultural factors.

This brief review of the causes of sex differences leads to a logical series of questions that should be answered in a comprehensive investigation of the origins of sex differences in any brain or behavioral trait in mammals.

Designs and Methods for Research on Sex Differences

The same fundamental questions and experimental design issues apply across a broad range of content areas that researchers might choose to investigate (see Fig. 1). Theoretical considerations as well as practical methods for the competent study of sex differences are discussed in the following sections. For each question we give examples of studies that have taken the approach or used the treatments being discussed.

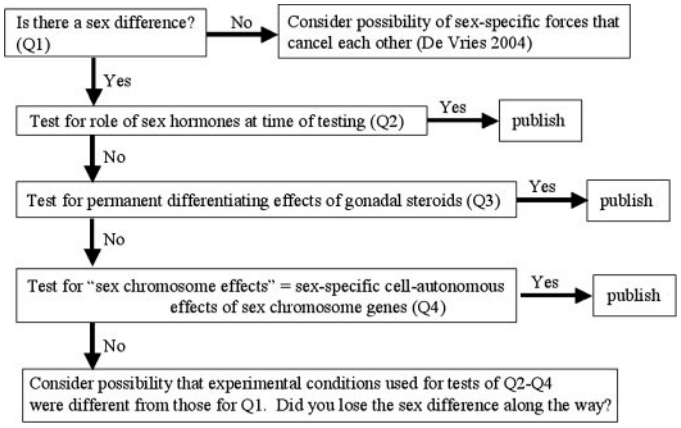

FIG. 1. Logical series of experimental questions. The figure illustrates an ordered and logical series of questions that can guide the investigator who wishes to understand the biological origins of sex differences in a trait. The questions and methods to answer them are explained in greater detail in the text. If a sex difference is detected in gonad-intact animals, the most likely reasons are sex differences in the effects of gonadal hormones around the time of testing (Q2), or permanent differences caused by the action of gonadal hormones during fetal or neonatal life (Q3). If gonadectomized males and females are tested after being given equivalent hormone treatments and there is a sex difference, then it is possible that there is both a role for sex hormones around the time of testing and a permanent differentiating effect of gonadal steroids on the trait, in which case even if the answer to Q2 is yes, it is still appropriate to ask Q3. Q, question.

Question 1: is there a sex difference in the trait?

One might think that to study sex differences, a two-group design in which one simply measures the trait in a group of males and a group of females and tests whether the two groups differ is sufficient. To compare males and females, however, one must determine the age at which testing will occur, consider the environments in which they have developed, the time of day of the testing, the appropriate method of measurement of the trait, *etc.* In short, knowledge of the individual's biology is important as well as a knowledge of the environments and testing situations that would be optimal for testing for a sex difference. Some sex differences occur only in certain environments, at specific ages, or at particular stages of the reproductive cycle. If one finds a group difference in a trait in males *vs.* females, one can conclude that the there is a sex difference in the trait at least when it is measured under those conditions. For practical purposes, it is often of interest to determine whether there is a sex difference in adults of reproductive age. It would be an error, however, to simply assume the observed sex difference will generalize to other ages or conditions.

There are occasionally dramatic effects of ovarian steroid hormones on sexually dimorphic traits, so it may not be sufficient to measure females at random throughout the reproductive cycle. In fact, many traits will differ as a function of day of the female's reproductive cycle. A better approach is to compare males with two or more groups of females

where the stage of the ovarian cycle is known. A three-group design in rats, for example, could compare males with females on two specific days of the estrous cycle. A five-group design would compare males with females on each of the four days of the rat estrous cycle (*e.g.* Ref. 17). The results of the study would show whether males and females differ on the trait and, if so, on which estrous cycle days (see Question 5). This experimental design has the potential to indicate whether actions of gonadal steroids at the time of testing could influence the dependent measure. Because other variables co-vary with the ovarian cycle, any estrous or menstrual cycle effects that are found need to be verified in follow-up studies in which hormone concentrations are experimentally manipulated to determine the role that the specific hormones play in affecting the dependent variable (see Questions 2 and 6).

If a trait varies with the ovarian cycle, it is by definition a sexually dimorphic trait because males will not show the same cyclical variation (because they don't have an ovarian cycle). With a simple two-group design discussed in the last paragraph, if females differ from males on some, but not all, days of the estrous or menstrual cycle when females are selected randomly, they may not be randomly distributed across the cycle, and a sex difference could be missed. This is particularly true if the sample size is small or if all females are measured on the same day, because female rodents, humans, and other mammals that spend a lot of time together may synchronize their cycles (18–20). Even if females used in an experiment are at different stages of the ovarian cycle, a sex difference could be missed because of the variability among the animals in the group.

Measuring a trait in gonadally intact males and females is important because it gives information on the trait measured under natural conditions, with all endocrine feedback systems operating and all natural gonadal secretions operating. The presence of a sex difference, however, does not tell you which hormones, if any, are important for their effects on the trait being measured.

Question 2: is the sex difference attributable to the actions of gonadal steroids at the time of testing?

The following sections discuss methods used, first in laboratory animals (primarily rats and mice) and then in humans.

Laboratory animals. Once a sex difference is detected, the next step is to determine whether gonadal secretions cause or affect the difference by acting just before or during the time of testing (see Fig. 1, Q2). In studies with laboratory animals, the classical method to answer this question has two stages, endocrine ablation and hormone replacement therapy. First, the gonads of males and females are removed (controls are operated on and the gonads are externalized and then replaced, a procedure called sham gonadectomy), and the trait is measured. Depending on the trait of interest, it may be necessary to wait days or weeks for the effects of gonadal secretions to wear off. Indeed, the time after gonadectomy can be varied to determine how fast the effects subside, if this is relevant. If gonadectomy changes the trait in one sex, then one concludes that the gonads are important for the trait in that sex (*e.g.* Ref.21). One cannot conclude strictly at this stage that gonadal secretions influence the trait because other factors (*e.g.* neural afferents from the gonad) are also manipulated by gonadectomy. If gonadectomy abolishes the sex difference, then by Occam's razor there is no need to invoke any other factors (*e.g.* permanent masculinizing effects of testosterone during fetal life) as the source of the sex difference. If gonadectomy abolishes the sexual dimorphism, it is still possible that two sex-specific factors, such as hormonal and genetic factors, operate in a sex-specific fashion to counteract each other, reducing rather than producing sex differences in phenotype (22).

If gonadectomy in nonhuman animals changes the trait in one sex, the second step is to administer gonadal steroids to gonadectomized animals (controls receive placebo treatment) to determine whether the gonadal steroids cause the trait to return to its natural state (*i.e.* the state typical of gonadally intact animals). This experiment can be performed in a three-group design: gonadectomized animals given gonadal steroid, gonadectomized given placebo, and intact controls. If testosterone treatment in castrated males returns their phenotype to that of an intact male after a given time period, the result strongly supports the idea that testosterone from the testes influences the trait, directly or indirectly (*e.g.* Ref. 23). To reach this conclusion it is important that the concentrations of gonadal steroid be within the physiological range; otherwise, pharmacological effects of the hormone can be observed. It should also be noted that the effect of a hormone treatment can vary with the time after treatment, because steroid hormones can have both rapid effects as well as effects that are not seen for days or months. So, choosing appropriate time points is another thing to think about when designing the experimental protocol. Another important decision is which gonadal hormones to replace. Depending on the specific parameters of the experiment, one might wish to replace the male's hormones (*e.g.* testosterone) or the female's (*e.g.* estradiol or progesterone alone or in combination) (see Question 6).

There are numerous variations on the classic two-step endocrine experiment. Various drugs are available to block specific gonadal steroid receptors or the synthesis of steroids to test the influence of steroid hormones on the trait. Examples are flutamide, a well-established and effective androgen receptor blocker (*e.g.* Ref. 24); tamoxifen, an estrogen receptor antagonist (*e.g.* Ref. 25), and RU486, an effective anti-progestin (*e.g.* Ref. 26, 27). Fadrozole is an effective inhibitor of aromatase, the enzyme catalyzing 17β-estradiol synthesis from testosterone (*e.g.* Ref. 28). Discussion of the huge variety of steroid hormone agonists and antagonists is beyond the scope of this paper. It should be noted, however, that use of these drugs requires some research on the specific application before beginning; we have given examples of studies that have used these agents as a guide. For example, tamoxifen is an estrogen receptor antagonist in some systems (breast and uterus) and an agonist in others (bone), and RU-486 is active at glucocorticoid receptors as well as at progestin receptors.

Since the early 1990s, mice have been generated that have a null mutation (knockout) of specific steroid receptors. When these mice are compared with control mice of the same

genetic strain, the influence of a specific steroid receptor can be measured (*e.g.* the Tfm mouse that is lacking androgen receptors and so appears feminine even if the animal has testes). Knockout mice have allowed the identification, for example, of previously undetected estrogen receptor-dependent effects on specific traits (*e.g.* Ref. 29). The knockout mice, however, do not represent animals in whom all effects of a specific hormone have been eliminated, because other steroid receptors are often functional in these animals, and nearly all of the gonadal steroids also act via mechanisms other than those mediated by their classic receptors. Moreover, when comparing knockout with wild-type mice, the differences are attributable to the loss of hormone action in adulthood and/or throughout development, because receptors are absent throughout life. With the advent of conditional knockout mice, in which a receptor can be inactivated at specific life stages and/or in selected tissues, it should become possible to specify more clearly the site of action of the hormone and the life stage at which the trait is influenced by the hormone acting through specific receptors.

Humans. In humans, one can reduce the variability that stems from fluctuating hormone concentrations associated with the menstrual cycle by measuring ovarian steroids at the time of study and classifying women into groups accordingly (*e.g.* (30). Classifying women by stage of the menstrual cycle defined on the basis of timing or indirect criteria such as changes in basal body temperature is a less desirable alternative, because there are large interindividual differences in the hormone concentrations attained at each stage. In addition, self-reports of menstrual cycle stage are notoriously unreliable. Because the menstrual cycle is only a rough index of the hormonal profile, it is therefore better methodology to directly measure ovarian hormones, unless for theoretical reasons the menstrual cycle itself is of interest (e.g. when studying the timing of mood changes linked to the cycle) or when there is a need to prospectively estimate the timing of future endocrine events.

Women using hormonal contraceptives must be considered separately, because the contraceptives greatly alter the endocrine environment in ways that depend on the specific contraceptive formulation. Studying females in different endocrine states or choosing one endocrine state and comparing it with males matched on specific criteria (*e.g.* age, education, socioeconomic status, race, *etc.*) will aid in determining factors that contribute to variability between women and within a woman as well as between women and men (see Questions 7–9).

As in females, hormone concentrations differ systematically in males as a result of puberty, adolescence, adulthood, and aging. In men, total testosterone concentrations in serum drop by approximately 35% and bioavailable testosterone by 50% between ages 30 and 80 (31). There are also circadian and seasonal variations in testosterone concentrations [this is true in many species including humans where testosterone is highest in the early morning and in autumn (32, 33)]. These biological rhythms need to be taken into account in designing studies, because the actions of testosterone at the time of testing might influence the sex differences observed. For example, it may be necessary to control the time of day at which measurements are taken or to use stratified sampling techniques.

In humans, trying to determine the cause of a sex difference is much more difficult. The gonads cannot be removed for experimental purposes. Therefore, we must rely on other methods. These might involve observing the effects of surgical removal of the gonads [*e.g.* surgical menopause (34), suppression of gonadal secretions as in men being treated with antiandrogens for prostate cancer (35), or the effects of replacement hormones, *e.g.* hormone replacement therapy (HRT) in postmenopausal women (36, 37)]. One issue is that such clinical manipulations are typically carried out in older persons. Whether aging is accompanied by any changes in receptors that might affect hormone responsivity is not known. Although still a valuable strategy, the applicability of the findings to pubertal girls and young women of reproductive age is unknown. Another issue is that pure hormonal preparations (*i.e.* those that contain a single hormone or that are the same as the hormone produced by the body) are not always used. Exposure to a mixture of estrogens, for example, is common in HRT. We will further discuss the methods used in human studies below (see Question 8).

Question 3: is the trait sexually differentiated permanently by the action of gonadal steroids during pre- and/or postnatal periods of development?

Laboratory animals. If gonadectomy around the time of testing (*e.g.* in adulthood) does not eliminate the sex difference in the trait, one then suspects that testosterone (or its metabolites 17β-estradiol or dihydrotestosterone) might have acted during early stages of development to cause permanent sex differences in development of the tissues mediating the trait (see Fig. 1, Q3). Another outcome suggesting this hypothesis is if gonadectomized males and females treated identically with gonadal steroid(s) show differences in the trait. The latter situation holds, for example, when the trait occurs only in the presence of gonadal steroids in one sex. For example, female sexual behavior in guinea pigs is abolished by ovariectomy but is reinstated in females much more than males when both are given 17β-estradiol followed by progesterone (38). In sum, permanent sex differences are suspected whenever males and females show a difference in a trait under conditions of equivalent concentrations of circulating gonadal steroids. So, if an investigator is following the sequence of experiments depicted in Fig. 1, there are instances when there will be a role for hormones at the time of testing (Q2), and one proceeds to Q3 to investigate whether there are permanent differentiating effects of gonadal steroids, because equivalent hormone treatments produced different effects in males and females.

Two general approaches are used to test the role of testosterone in sexual differentiation: increase testosterone exposure in females or reduce it in males. The first approach involves administering physiological doses of testosterone to females during fetal and/or early postnatal life (controls receive placebo) and measuring the trait later in life, usually in adulthood, to determine whether the female is more masculine by virtue of the testosterone treatment (*e.g.* Ref. 39). Often it is appropriate to include a placebo-treated male

control group for comparison. If testosterone treatment causes females to become more like males, it is possible to conclude that females can respond to testosterone and that testosterone can cause masculinization of females. To determine whether testosterone normally masculinizes the trait in genetically male animals, the classic endocrine experiment is performed (endocrine gland removal followed by hormone replacement) by manipulating exposure to testosterone before and/or just after birth. A typical approach to reduce the action of testosterone in prenatal males is to administer an androgen receptor blocker to the pregnant mother (24). These drugs pass through the placenta to the fetus. Because many of the masculinizing actions of testosterone in rodents require conversion of the testosterone to 17β-estradiol in the brain, estrogen antagonists or aromatase inhibitors are also given to block normal masculinization of traits in males. Controls receive placebo. After birth, the hormones are manipulated by removal of the testes (controls are sham gonadectomized). In rats, from gestational d 18 through the first 10 d of postnatal life encompass the critical periods where several aspects of brain masculinization and defeminization can occur (9, 10). The trait of interest is often measured some weeks or months later, under conditions appropriate to the trait.

If blocking or removing testicular secretions during fetal or postnatal periods prevents the development of a masculine trait in males, and testosterone (or 17β-estradiol) treatment prevents this demasculinization of males and causes masculinization of females, then the trait is sexually differentiated by the actions of testosterone during the early developmental period tested. The effects of testosterone are typically permanent. They can be measured many months after the endocrine manipulation, long after the steroid hormone itself is no longer present (9, 10).

Often administering testosterone to females causes incomplete masculinization, in that the trait in testosterone- or 17β-estradiol-treated females is intermediate to that of normal males and females. The explanation for the incomplete masculinization of females usually is that the testosterone treatment does not fully mimic the normal patterns of concentrations or timing of hormone exposure found in males. For example, testosterone treatment during the perinatal period does not produce complete masculinization of the phallus in female rodents (7, 10). Similarly, if antisteroids and/or postnatal castration do not completely prevent masculine development in males, the investigator might conclude that the reduction in testicular secretions does not fully mimic the endocrine state of the developing female. In each case, the failure to fully cause or prevent masculine development is not necessarily taken as strong evidence for the importance of other factors (other hormones or nonhormonal factors) to cause the sex difference.

Experiments in which the serum concentrations of gonadal steroids are manipulated allow strong conclusions about the effects of gonadal steroids during specific developmental periods but do not necessarily answer the question of whether the sex difference is entirely explained by gonadal secretions. In cases in which treatment of females with physiological concentrations of testosterone cause complete sex reversal (*e.g.* Ref. 40), there is no need to invoke other possible causes of the sex difference (41).

In general, experiments such as these show strong effects of testosterone or in some species its metabolite 17β-estradiol, which act on the brain to cause masculine patterns of development. A role for ovarian secretions in bringing about feminine development of the brain has also been suggested, but the effects of ovarian secretions appear to be more subtle, perhaps more limited, and to act in a different time frame during development (42). The same experimental approaches described for testosterone above are appropriate for manipulating the concentrations of ovarian hormones during various phases of development to determine their importance in causing sex differences.

Humans. The classic experimental approach involving gonadectomy and manipulation of hormones cannot be applied in human studies. However, sexual differentiation of the human nervous system and other tissues under the influence of testosterone might well occur. One approach used in humans is to study the behavior and physiology of females who were exposed to high concentrations of androgens prenatally as a result of endogenous overproduction of androgens by the adrenal glands. This occurs in genetic females with the classic form of 21-hydroxylase deficiency, or congenital adrenal hyperplasia (CAH). In this condition, excess androgens are produced *in utero*, but once cortisone replacement therapy is initiated, usually in the first few weeks after birth, androgen concentrations become normalized. Therefore, females with CAH are exposed to high concentrations of androgens during prenatal but not postnatal development. Accordingly, this is the best available model in humans for studying the effects of prenatal androgens on sexual differentiation. Psychosexual development and gender role behaviors have been the focus of most studies (43, 44), but the effects of CAH on patterns of gonadotropin secretion, menstrual cyclicity, and other features of female reproductive physiology could also be studied.

Although such individuals are rare, it is also possible to study genetic males who have androgen insensitivity syndrome for insights into the role of androgens in sexual differentiation of the nervous system. As in other species, the syndrome is caused by absence or malformation of the androgen receptor, resulting in an inability of the tissues to respond to androgens. The insensitivity may be partial or complete. In practice, the study of males with androgen insensitivity syndrome is complicated by the fact that they are raised as females and typically take estrogen supplementation (10).

Question 4: do sex chromosome genes act in a cell-autonomous fashion to cause the sex difference?

In some cases a sex difference may appear not to be explained by the activational effects of gonadal secretions during the time of testing (*e.g.* adulthood) or by organizational effects during pre- and postnatal development. If manipulations of sex steroid hormones around the time of testing, and during fetal and neonatal life, all fail to sex-reverse the trait, then other factors become more interesting. Several explanations are possible. One is that gonadal steroids are

responsible for the sex difference, but they act at a developmental stage that has not yet been tested. In some cases, for example, steroids acting during puberty may have persistent effects on traits measured later in life (45, 46). So far, little is known about the potential for pubertal effects in humans. Of course, the lack of effect of a hormonal manipulation in animal studies does not prove that hormones are not involved, because the experiments may have failed to manipulate the hormones effectively (*e.g.* dose too high or too low, wrong metabolite used, insensitive measurement of the dependent variable, *etc.*).

Laboratory animals. Another potential explanation is that genes encoded on the sex chromosomes (X-linked or Y-linked genes) act within cells to sexually differentiate XX and XY cells (Fig. 1, Q4). In this article, this kind of effect is defined as a sex chromosome effect. This mode of sexual differentiation is well established in many tissues in invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* (47) and in the mammalian gonad (48). Specific X and Y genes are implicated in each of these cases. In a few cases, this mechanism also operates in nongonadal tissues in mammals (49–53). In all of these mammalian cases in which a sex chromosome effect is inferred, the sex difference can be detected before differentiation of the gonads. Hence gonadal secretions (*e.g.* testosterone, Müllerian-inhibiting factor, IGF-3), which are first secreted after the gonads differentiate, cannot be responsible for the initial stages of sexual differentiation of the tissue, and a cell-autonomous sex chromosome effect is implicated. In each mammalian case, however, the specific X- or Y-linked genes responsible have yet to be identified.

To test for a sex chromosome effect to explain sex differences in a trait that develops after gonadal differentiation, when hormones cannot easily be dismissed as a contributing factor, the basic approach would be to manipulate the dose of X and/or Y genes (or allelic differences in those genes) while keeping the concentrations of sex hormones as constant as possible. For example, one could detect a sex chromosome effect by finding a sex difference in a trait in XX *vs.* XY animals if the gonadal secretions were either entirely absent throughout life or were held scrupulously constant. In practice, this has been achieved rarely if at all. Manipulation of gene expression in an intact mammal is currently economical and routinely feasible only in mice and is much more difficult than manipulation of hormone concentrations. Here we describe one experimental approach pioneered by Burgoyne, Lovell-Badge, and colleagues (49, 54, 55).

A strain of mouse exists in which *Sry*, the testis-determining gene, is deleted on the Y chromosome. This Y^- chromosome therefore does not induce testis development. Instead, XY^- animals have ovaries and are called females (male and female here are defined by gonadal phenotype). Some mice carry an *Sry* transgene inserted into an autosome making the mouse XY^-*Sry*. Phenotypically, the animal is male (with testes) and breeds normally. When XY^-*Sry* males are mated to XY^- females, four types of progeny result: XY^-*Sry* males, XX*Sry* males, XY^- females, and XX females. These are called the four core genotypes. If one measures traits in these four genotypes, the comparison is 2×2 (the factors are gonadal sex and sex chromosome complement). One can discover phenotypes that are influenced by hormonal secretions of the gonads by comparing females and males (either XX females *vs.* XX*Sry* males, or XY^- females *vs.* XY^-*Sry* males). These two comparisons keep the genetic sex of cells constant but vary the sexual phenotype of the gonads (ovaries *vs.* testes). The other comparison keeps the sexual phenotype of the gonads constant (almost constant, see caveat below) while varying the genetic sex of cells throughout the body: XY^- females *vs.* XX females; XY^-*Sry* males *vs.* XX*Sry* males (56).

For example, if one measures the size of brain nucleus A in the four core genotypes and finds that A is larger in male groups than in female groups (XY^-*Sry* > XY^- and/or XX-*Sry* > XX), the difference is attributable to the action of *Sry* (41). The most likely sites of action of *Sry* to cause a difference in region A are the gonads because of the well-known role of gonadal steroids in causing sex differences in the brain and other somatic tissues. As a shortcut, these comparisons are said to test for a role of gonadal hormones. However, this experiment does not rule out an effect of *Sry* elsewhere in the body, for example in the brain itself. (Logically, such an effect is possible, although presently we know of no such actions of *Sry* outside the gonad.) On the other hand, if one finds that region A is (for example) larger in XY^- mice than XX mice within sex (XY^-*Sry* > XX*Sry* and/or XY^- > XX), then the conclusion is that the complement of sex chromosomes of cells causes the group differences. For a more specific example of this type of cell autonomous sexual differentiation of the brain, see Ref. 41. This result does not indicate which genes are responsible or where they act. They might act, for example, inside or outside of the brain. The result does not prove that the sex chromosome effect is nonhormonal because some hormonal mechanisms could conceivably be involved. Hypothetically, if the adrenals of XY^- females secreted more androgen than those of XX females, the sex chromosome effect could be mediated by androgens. In the long run, the mechanisms of action caused by sex chromosomes can be elucidated by identifying the genes responsible and studying their cellular site and mechanisms of action. Finally, although the two male groups will differ strikingly from the two female groups in their gonadal secretions, the two male groups (or two female groups) may differ more subtly from each other in the concentrations of hormones secreted by the gonads. Despite this possibility, the two male groups have been found not to be different from each other, and the two female groups are not different from each other, as assessed by measuring several brain regions and behaviors that are sensitive to threshold concentrations of gonadal hormones (41, 57). Therefore, the within-sex differences in gonadal secretions are likely to be minor.

This example illustrates that the genetic sex of cells (XX *vs.* XY) can now be manipulated independently of the sexual phenotype of the gonads, to begin to dissociate the effects of sex chromosome genes and gonadal steroids. These studies are currently feasible only in mice, but we can expect improvements in molecular genetics to allow similar experiments in other species. Other mouse models also exist, for example knockouts of genes such as steroid factor 1, which are required for gonadal development (58). These models

allow measurement of responses in mice that lack gonads entirely.

Research Issues in the Study of Hormone-Behavior Relations in Adults

This section includes more specific questions and issues that investigators face when studying hormone-behavior relations within the context of sex differences research.

Question 5: how is the stage of the estrous cycle determined for the female rat?

Ovarian cyclicity has profound effects on most, if not all, of the nervous system. In rats, as well as other species, these effects produce transient changes in behavior. For example, reproductive behavior, food intake, fluid intake, and locomotor activity vary markedly with stage of the estrous cycle in female rats (59–61). The study of estrous cycle effects represents an opportunity to study neuronal plasticity and its physiological and behavioral consequences, yet many investigators remain wary because published protocols for assessing estrous stage seem inconsistent and unclear. This issue is addressed by describing two of the main strategies used for assigning the rat's estrous stage in relation to a light-dark cycle. Behavioral endocrinologists frequently use a 14-h light, 10-h dark cycle or a 12-h light, 12-h dark cycle (see below).

The female rat is a spontaneously ovulating polyestrous mammal. Ovulation occurs at 4- or 5-d intervals throughout the reproductive life of the rat, except during pregnancy, pseudopregnancy, and lactation (60–62). 17β-Estradiol and progesterone are the hormones produced in the largest quantity by the ovaries. During the estrous cycle, 17β-estradiol and progesterone act on the brain to stimulate the hormonal events that result in ovulation and sexual behaviors (63). These hormones also act on peripheral receptors and glands to induce the production of pheromones by the female rat. Therefore, these hormones will produce qualitative changes in the value of a female as a stimulus in addition to changing her behavior. For example, 17β-estradiol enhances whereas progesterone decreases attractiveness of the female rat to a male rat because of changes in the female's pheromone production (64). For sexual behavior, 17β-estradiol primes the brain for progesterone by inducing the production of progesterone receptors, and then progesterone activates sexual receptivity (63).

As mentioned above, female rats have only a 4- or 5-d estrous cycle (Fig. 2B). This is one of the most rapid ovarian cycles among mammals, and it is made possible by truncating the cycle after ovulation. In other female mammals, ovulation is followed by a luteal phase, which is maintained by hormones produced by the corpus luteum (a transient endocrine gland comprising the residual components of the follicle that remain after ovulation) (63). In rats, the corpus luteum becomes functional only when the female engages in sexual behaviors that activate a progestational reflex that prolongs the secretion of progesterone from the corpus luteum. This reflex induces the release of prolactin from the anterior pituitary. Prolactin in turn stimulates the corpus luteum, which in turn secretes progesterone to prepare the uterus for implantation, and this increases the probability that pregnancy will occur. Stimulation of the vaginocervical area by the male rat or the experimenter can induce the progestational reflex, resulting in a period of corpus luteum activity that lasts 12–14 d. This condition is known as pseudopregnancy (60, 65).

The estrous cycle. When the aim of an experiment is to determine whether an outcome measure varies with the stage of the estrous cycle it is important to determine whether animals are cycling normally. Thus, it is prudent to establish that at least two complete cycles have occurred before initiating testing.

Follicular phase. The ovarian cycle begins with the development of follicles from oocytes in the ovary. Low concentrations of FSH from the pituitary stimulate follicular development. There is also increased steroidogenesis at this phase caused by stimulation by LH. 17β-Estradiol secretion increases gradually during this phase. In the rat, this stage is 2 d long. The first day is called diestrus 1 or metestrus, and the second day is diestrus 2 or just diestrus (Fig. 2B) (63).

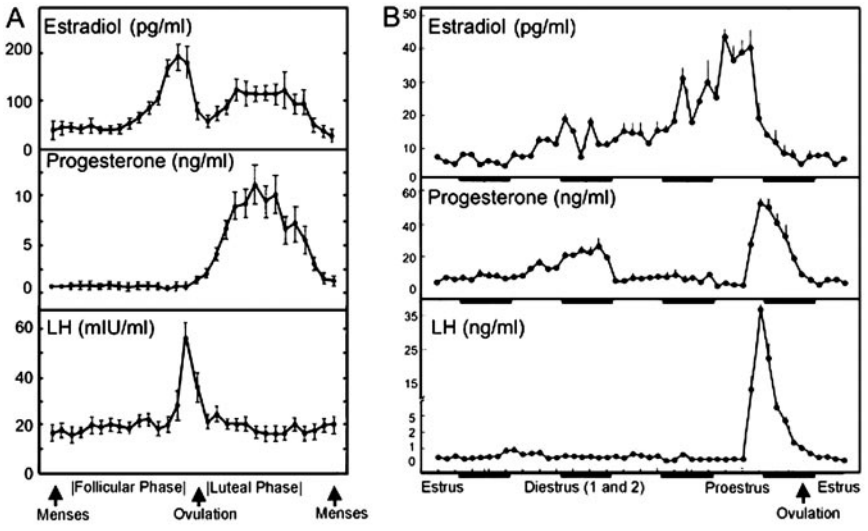

FIG. 2. Patterns of estradiol, progesterone, and LH inhuman (A) and rat (B) during the reproductive cycle. Time unit of the x-axis in A is days; in B, it is hours. *Dark bars* in B indicate dark period of the day/night cycle. Note that during the follicular phase in humans and its analog in rats (diestrus), 17β-estradiol rises but progesterone secretion remains low. After the LH surge, progesterone is elevated in both rat and man. In humans, the corpus luteum also secretes some 17β-estradiol, whereas in rats during the brief luteal phase, 17β-estradiol concentrations decline. [Panel A is adapted with permission from Fig. 4 in I. Thorneycroft *et al.*: Am J Obstet Gynecol 111:947, 1971 (128). Panel B is adapted with permission from Fig. 1 in M. S. Smith *et al.*: Endocrinology 96:219, 1975 (201). © The Endocrine Society.]

Periovulatory period. The time just before and after ovulation is dynamic. 17β-Estradiol increases dramatically, acting on the brain to trigger GnRH release, which induces a surge of LH from the pituitary that induces ovulation. Progesterone rises a few hours before ovulation and contributes to this process. In the rat, this phase is called proestrus (63). Maximum 17β-estradiol release from the ovary starts 18 h before ovulation with serum 17β-estradiol reaching a peak of 50–150 pg/ml around 6–12 h before ovulation (60, 65). A significant increase in progesterone occurs 4–6 h after the 17β-estradiol surge, during the afternoon of proestrus. The serum concentration of progesterone at the proestrus peak is approximately 25–50 ng/ml (60, 65). Once LH and progesterone are released into the circulation, ovulation occurs 10–12 h later (Fig. 2B).

Estrous phase. Estrus is the period of sexual receptivity and the actual day of ovulation. Sexual receptivity onset occurs shortly after the onset of the dark phase of the light-dark cycle and precedes ovulation by a few hours in most animals (Fig. 3). Ovulation, induced by the LH surge on proestrus, occurs 10–13 h after the surge (66), and sexual receptivity persists for 12–20 h (depending on whether the female mates or not). Note that behavioral receptivity occurs 36–48 h after the initial increase in 17β-estradiol and 4–6 h after progesterone. Baseline serum concentrations of 17β-estradiol at vaginal estrus or behavioral estrus are approximately 3–12 pg/ml (60, 65).

Further details of the stages of the estrous cycle and the endocrine changes that accompany them can be found in Ref. 67 for the rat, hamster, guinea pig, sheep, dog, and rhesus monkey.

Nomenclature. Clearly, the estrous cycle is a dynamic process with changes happening rapidly, particularly during proestrus and estrus. This can lead to some confusion and misunderstanding about findings if the dynamic nature of the process is not taken into consideration. For example, the hormonal profile during the morning of proestrus is very different from on the afternoon of proestrus (60). Furthermore, confusion can occur because of different nomenclatures that can be used to determine when a day begins and ends. Behavioral neuroendocrinologists, for example, frequently define a day of the cycle as beginning with the time that lights go off in the animal colony so that the entire active period of the animal's subjective day falls on one day of the estrous cycle (see Fig. 3). Other scientists may define a day as beginning at midnight to be consistent with the scientist's subjective day. Given the dynamic nature of the cycle, it doesn't really matter which nomenclature is used as long as

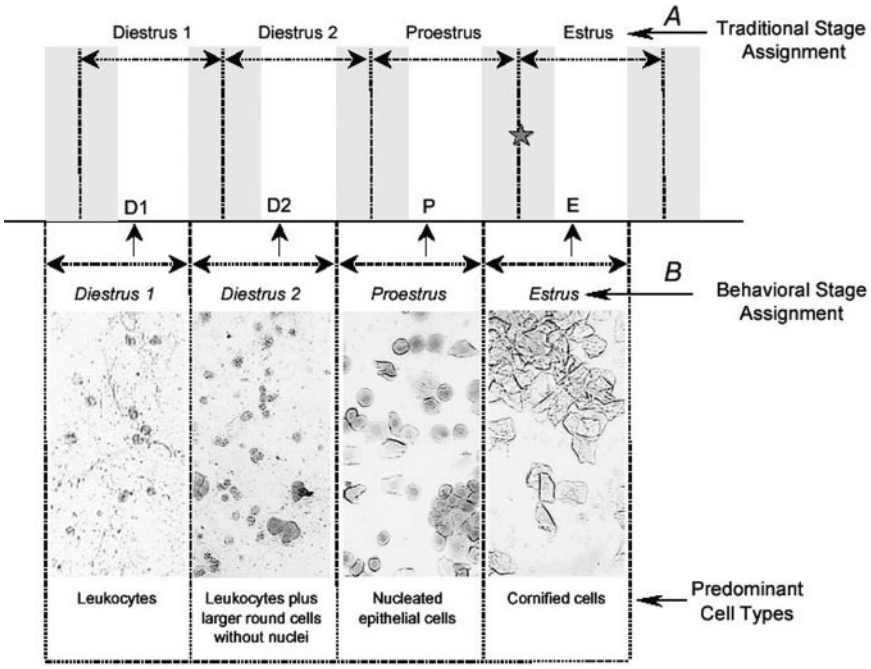

FIG. 3. Stage assignment across the rodent's 4-d estrous cycle in relation to a 12-h light, 12-h dark cycle and samples of vaginal cytology. *Shaded bars* located in the upper portion of the figure denote successive 12-h dark periods. *Vertical arrows* depict the time (early- to mid-light phase) when vaginal cytology is typically sampled. The *gray star* denotes the time of ovulation (4–6 h into the dark phase). Representative photomicrographs and a brief summary of the types of cells that are predominant during each cycle stage are depicted in the lower portion of the figure. Examination of vaginal cytology reveals that stages of the estrous cycle range from 8–54 h. By convention, however, scientists refer to stages of the estrous cycle in relation to our circadian day. A, Traditional stage assignment. Each successive 24-h interval from mid-dark period to mid-dark period is named for one stage, consistent with the scientist's subjective day (60, 65). B, Behavioral stage assignment. This strategy considers the rodent's behavioral traits that are expressed predominantly during the four successive dark periods. For example, changes in sexual receptivity, food intake, and locomotor activity occur primarily during the dark period when rodents ovulate (59). Using the traditional strategy for assigning estrous cycle stage, this period of altered behavioral expression coincides with the end of proestrus and the start of estrus. The ambiguity created by these behavioral changes spanning two estrous cycle stages has created confusion in the literature. To avoid this problem, we propose that the 12-h dark period, coincident with ovulation, be referred to as behavioral estrus. To ensure comparison between studies we also propose that scientists explicitly state assignment of estrous stage in relation to the light-dark cycle as well as the endocrine events occurring during the time of experimental investigation.

it is defined and results are interpreted relative to the endocrine events happening at the time of the experimental manipulations.

Determining estrous cycle phase by vaginal lavage. For research purposes, it is generally preferable to measure hormone concentrations in the serum directly if inferences are to be made about hormone concentrations. However, hormonal changes associated with the ovarian cycle result in cellular changes in vaginal cytology. For some research purposes (*e.g.* verifying whether an experimental treatment has disrupted the estrous cycle) it may be sufficient to monitor changes in hormones indirectly by monitoring changes in vaginal epithelial cells. Most investigators assign estrous stage using the relatively noninvasive technique of examining vaginal cytology at 24-h intervals. Using this technique, it has been determined that the estrous cycle consists of four stages named diestrus 1 (D1, sometimes referred to as metestrus), diestrus 2 (D2), proestrus (P), and estrus (E). Each of these stages has varying lengths (D1, ~6–8 h; D2, ~55–57 h; P, ~12–14 h; and E, ~25–27 h), and the specific changes depend on the species and the light-dark schedule. If samples are obtained at 24-h intervals, not all of these stages may be sequentially visible in a set of smears from an individual rat, because some stages are less than 24 h (60). Note, also, that even though the nomenclature is the same, these terms do not refer to the same 24-h periods identified in Fig. 3 and in the paragraphs above. For example, the vaginal cytology characteristic of proestrus lasts 12–14 h, not the 24 h associated with the day of proestrus as identified above. Finally, the vaginal cytology does not immediately reflect changes in hormonal secretion, because there is a delay between hormonal secretion and the morphological changes in the vaginal target cells.

As illustrated in Fig. 4, vaginal lavage is usually done using an eyedropper and 0.9% saline. The tip of the eyedropper is filled with a small amount (one or two drops) of saline and then inserted into the vagina of the female rat (Fig.

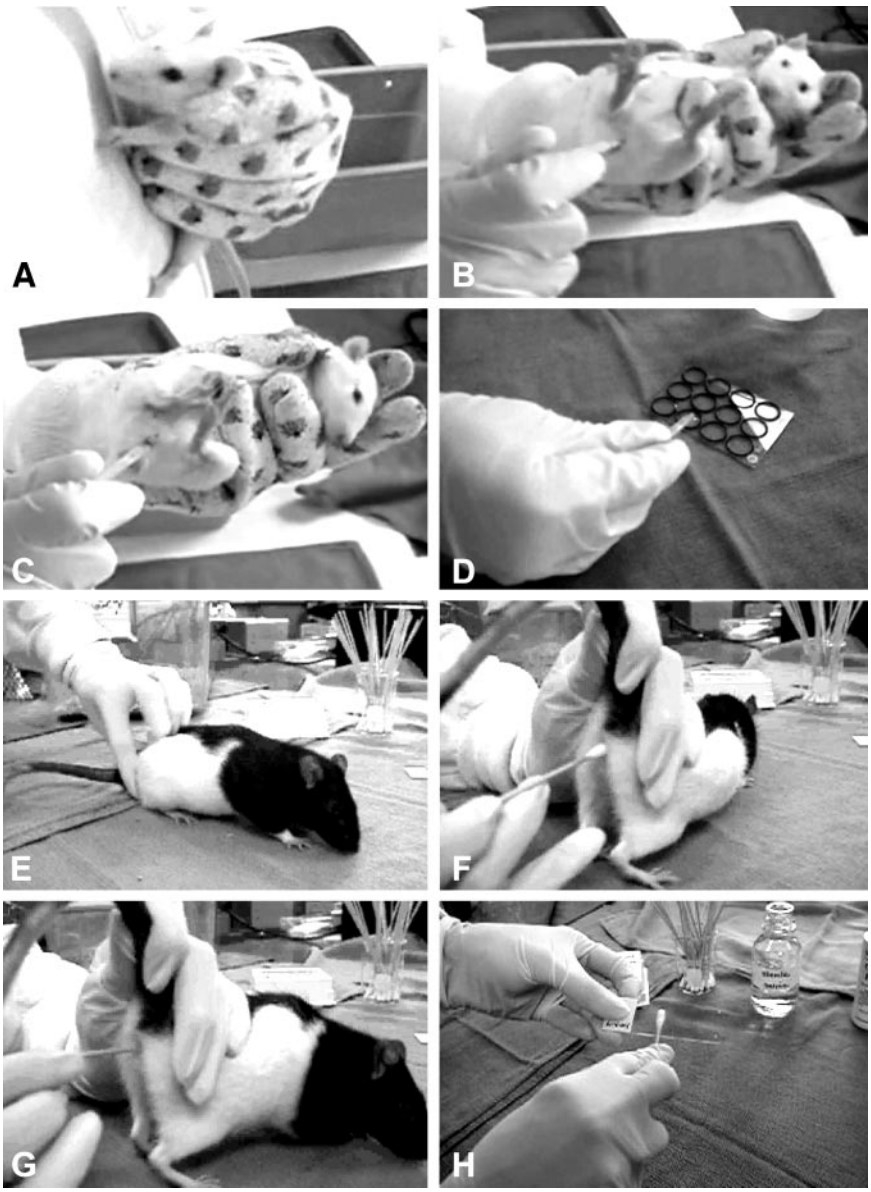

FIG. 4. How to obtain vaginal cells for cytology. Depicted are two methods commonly used to perform vaginal lavage. The photographs shown here are single images taken from videos of the full procedure. A–D, Ventral approach in which the rat is first taken from the cage with the left hand, held against the chest briefly (A), and then turned ventral side up (B). Using the right hand, a blunt-tipped eyedropper is used to insert and quickly withdraw approximately 0.5 ml (maximum) of saline into the vaginal canal (C). The drop is then placed on a slide (D) so that vaginal epithelial cells that have sloughed off into the drop can be viewed later under a microscope. E–H, Dorsal approach. In this case, the rat is removed from the cage and placed on a table. Using the left hand, the rat is then grasped by the base of the tail (E), which is then lifted to expose the rat's vaginal opening (F). A cotton-tip applicator (moistened with distilled water or saline) is then inserted into the vaginal canal (F and G) and twisted rapidly and gently to accumulate epithelial cells. The cells on the applicator are transferred onto a slide by pressing the cotton tip onto the slide and vigorously rolling the tip to its full circumference (H).

4, A–D). The fluid is expelled into the vagina and then collected two to three times, or until the saline becomes opaque, and then the fluid is placed onto a microscope slide. The eyedropper is thoroughly rinsed with distilled water, and the next rat is sampled. The resulting sample is examined under a light microscope (×40–100 magnification), while still wet. Alternatively, a cotton swab, moistened with 0.9% saline, may be inserted into the vaginal canal and then rapidly withdrawn (Fig. 4, E–F). The resulting vaginal smear is transferred to a glass slide and examined under a light microscope at the same magnification (×40–100). Figure 3 describes the changes in cell types characteristic of the four estrous cycle stages in the rat.

For both strategies it is important that care is taken to avoid stimulating the cervix. If the eyedropper or cotton swab stimulates the cervix with enough pressure during proestrus or estrus, it can induce pseudopregnancy and, therefore, disrupt the rat's estrous cycle for approximately 12 d. Vaginal cytology should be examined daily, at the same time each day. Examination of these cells can be used to determine the cycle length and regularity of multiple cycles for each rat.

Reading the slides. It is not necessary to stain the cells, because the cells are sufficiently opaque to be visualized under a microscope with a blue or green filter. Alternatively, cells obtained via the vaginal smear technique may be fixed with ethanol (*e.g.* Surgipath cytology spray; Surgipath Medical Industries, Inc., Richmond, IL) to preserve the sample. Table 1 provides a protocol for staining the preserved samples for photographic purposes. The cytology sample can be viewed under a ×40–100 objective with a blue or green filter. Interpreting the stage of estrous cycle from the cell morphology requires practice and experience with a particular rat. The precise cellular morphology on a particular day of the cycle varies with the time of day (especially on proestrus). Rather than trying to guess what stage of the estrous cycle a female rat is in, describe the cell types that appear in the smear. After 5–6 d, it is usually possible to determine what that rat's smear looks like on estrus because of the abundance of cells usually obtained from lavage and their unmistakable appearance. Collect data for at least two complete cycles before determining where a female rat is in the cycle. This same method may be used to monitor the estrous cycle of mice, but the cells have slightly different characteristics (68–70).

TABLE 1. Protocol for staining vaginal smears

Step	Procedure
1	Dip fixed slides in 95% ethanol
2	Stain in Harris hematoxylin solution for 2.5 min
3	Dip several times in tap water
4	Dip twice in 0.25% hydrochloric acid
5	Dip in distilled water
6	Place in saturated lithium bicarbonate solution for 30 sec
7	Dip in distilled water
8	Place in 95% ethanol for 30 sec
9	Stain in eosin Y solution for 45 min
10	Dip twice in 95% ethanol
11	Coverslip slides

Question 6: which forms of the hormones should be given to rodents and by which route of administration?

Just as there are numerous choices in hormone replacement therapy for women lacking ovarian steroid hormone secretions, there is a wide variety of choices and variables (form of hormone, time, route, length of administration, *etc.*) for hormone replacement in research animals, whose ovaries have been removed experimentally. In this section, we discuss some of the hormone replacement procedures that have been used in rodents, and the rationales for selecting among them are discussed. Hormones administered to induce sexual receptivity and other physiological responses in female rats are used as examples. In the many studies on the hormonal regulation of sexual behaviors, the many facets of hormone administration have been extensively studied.

The choice for hormone replacement after gonadectomy must be guided by consideration of a variety of factors depending on the goals of the study. Is the goal of the study to 1) determine the hormones involved in a particular response during the estrous cycle or during pregnancy, 2) determine whether there is a sex difference in a particular response that is already known to be influenced by the estrous cycle, 3) determine whether gonadectomized females respond to a treatment that has a particular effect in gonadectomized males, or 4) assess whether a particular cellular mechanism of action of a hormone shows a sex difference in response?

Nomenclature (see also the glossary at the end of this article). There are two primary classes of ovarian hormones. Estradiol (also known as 17β-estradiol) is the principal circulating hormone of the class of hormones known as estrogens, and progesterone (also known as pregn-4-ene-3,20, dione) is the principal circulating hormone of the class known as progestins (or gestagens). Although 17β-estradiol is often referred to as estrogen, this usage is not technically correct, because 17β-estradiol is a specific hormone, whereas estrogen is a class of hormones. 17β-Estradiol is the most abundant circulating estrogen in vertebrates, so this is the particular estrogen that is usually administered in estrogen replacement in research animals. In males, the testicular hormone is primarily testosterone, an androgen.

Different estrogens. Although 17β-estradiol is typically the most physiologically active estrogen, this is not the only estrogen that has been used in endocrine or neuroendocrine research. For example, in a variety of *in vitro* systems, estriol has been compared in effectiveness to 17β-estradiol (71). In some behavioral experiments, estrone has been used rather than 17β-estradiol (72), perhaps in part because it is less effective than 17β-estradiol and less prone to inducing progesterone-independent feminine sexual behavior.

Routes of administration. 17β-Estradiol and progesterone can be administered by a wide variety of routes of administration. These include sc, iv, im, ip, and intracranial routes of administration. In some experiments, to alleviate the inconvenience of daily injections for long-term treatments, testosterone, 17β-estradiol, or progesterone, either in crystalline form (73, 74) or dissolved in peanut oil, has been enclosed in a small length of silicone tubing. It can also be delivered by an Alzet mini-pump (75) that delivers a consistent dose of

hormone for days, or by pellets supplied to deliver a particular dose of steroid hormone daily when implanted sc (76). Each mode of hormone replacement has its appropriate place, but different routes and regimens may provide discrepant results. In some cases, these differences in response to different treatments have been exploited to understand the hormones-behavior relationships better.

Esterified vs. free hormone. Because the circulating amount of 17β-estradiol does not remain elevated for very long after systemic injection of 17β-estradiol, slower-release, esterified forms of 17β-estradiol are often used in physiological research. A variety of esters and other modifications have been used, such as ethynylestradiol (77), estradiol valerate (78), or estradiol dipropionate (79). However, the most commonly used form is estradiol-3-benzoate, which is 17β-estradiol with a benzoic acid esterified in the third carbon position. This form is hydrolyzed *in vivo* to the physiologically active 17β-estradiol. Progesterone is injected only in an unmodified form (80). Testosterone is usually administered as testosterone or testosterone propionate (81).

In much of the early work in behavioral endocrinology, estradiol benzoate was the most effective form of estrogen found to induce sexual receptivity in females when followed by progesterone (80), so this became standard in the field of hormonal regulation of feminine sexual behaviors. It is likely that this form was chosen over free 17β-estradiol or estrone because it is generally effective in lower doses than either of these free, unesterified estrogens. Interestingly, though, many years after the pioneering work, others reported that free 17β-estradiol is more effective if given in a pulsatile fashion (82), for example, with two injections separated by at least 3–36 h (83).

The most typical mode of administration when attempting to induce lordosis behavior is one, two, or three daily injections of estradiol benzoate followed by progesterone. These treatments reliably induce the expression of feminine sexual behavior. In some cases, chronic daily injections of estradiol benzoate have been given without progesterone (84), although progesterone is essential for the facilitation of sexual receptivity during the estrous cycle (85) and for the full complement of sexual behaviors in rats (86). Although 17β-estradiol alone may be effective in inducing sexual behavior and in causing many of the behavioral changes seen during the estrous stage of the estrous cycle, the injection of daily 17β-estradiol or estradiol benzoate does not duplicate the preovulatory pattern of ovarian steroid hormones seen during the estrous cycle for two reasons. The preovulatory 17β-estradiol rises and falls over the course of a day and a half (See Fig. 2B), and the preovulatory 17β-estradiol is also followed by a surge in progesterone from the follicle and interstitium (87) (see Fig. 2B). Recently, Asarian and Geary (88) used a procedure in which a low dose of estradiol benzoate was injected sc every 4 d in ovariectomized rats. This treatment, which closely mimics the changes in 17β-estradiol concentrations over the estrous cycle, was effective in maintaining a variety of estrogen-dependent responses including responsiveness to progesterone facilitation of sexual behavior every 4 d, at rates similar to that seen during the rat's 4-d estrous cycle, suggesting that this regimen might be a very useful strategy for hormone replacement.

Silicone tubing capsules. When the treatment regimen requires or can accommodate prolonged exposure to the hormones, 17β-estradiol, progesterone, and testosterone have often been administered in the form of crystalline hormone implants in silicone tubing implanted sc (74, 89). Lipophilic steroid hormones dissolve through the wall of the silicone tubing and are released at a constant rate that depends on the surface area (length × diameter) of the capsule and the thickness of the capsule wall. Resulting hormone concentrations can be titrated by dissolving the hormone in oil vehicle (90) by diluting it with crystalline cholesterol (91) or by varying the size of the tubing itself. In addition, concentrations of circulating hormone remain rather consistent for an extended period of time (92) compared with injection of 17β-estradiol benzoate or another estrogen (93). To implant and remove silicone tubing capsules, the animals have to be anesthetized. However, they do not need to be handled daily as would be the case with injections. In some cases, silicone tubing capsules are inserted and removed at particular intervals, either to approximate hormone concentrations seen during the estrous cycle (90) or to provide the hormone in a more-or-less discontinuous pattern (94).

A similar mode of administration that has been used in mice is chronic implantation of silicone tubing capsules containing 17β-estradiol dissolved in oil (95). Often progesterone is injected weekly before tests for sexual receptivity. On the background of the chronic 17β-estradiol exposure, the weekly progesterone is quite effective. It must be noted, however, that although this might be useful in certain types of studies when prolonged elevation of 17β-estradiol concentrations is desired, the treatments bear no similarity to the patterns seen during the estrous cycle, as discussed earlier.

Pharmacokinetics. When replacing hormone by any of the modes of administration discussed here, pharmacokinetics must be taken into account. As would be expected, an iv, unesterified 17β-estradiol injection results in an immediate massive increase in blood 17β-estradiol concentrations, which decline with a time course dependent upon dose of hormone injected (96). On the other hand, a sc injection of the esterified estradiol benzoate induces a slower rise and fall of blood 17β-estradiol concentrations, typically lasting for one (97) or two (88, 98) days. A silicone tubing capsule implanted sc results in more of a square wave in 17β-estradiol concentrations with insertion and removal of the capsule (92), although the dynamics can be greatly influenced by whether crystalline 17β-estradiol (91) is used or 17β-estradiol dissolved in peanut oil vehicle (99, 100). Likewise, the hormone peak can be influenced by preincubation of the capsules before insertion (74, 101), the percentage of 17β-estradiol relative to cholesterol in the implants (91), and other factors.

Absence of hormone in blood is not absence of hormone. The decrease in blood concentrations of 17β-estradiol does not indicate that the 17β-estradiol is no longer active. 17β-Estradiol is retained by cell nuclear receptors for a considerable time after the decline in blood concentrations. For example, 18–24 h after an iv injection, 17β-estradiol is still bound by

cell nuclear estrogen receptors (96), functioning as transcription factors, long after circulating concentrations of 17β-estradiol have declined. Likewise, 17β-estradiol remains bound to estrogen receptors for at least 2 or 3 d after injection of a low dose of estradiol benzoate in rats (102) and guinea pigs (103), and the cellular and behavioral consequences of the hormone injection may persist for several days (102, 104), long after circulating concentrations of 17β-estradiol have decreased. In the case of another estrogen, 17α-ethynylestradiol, residual effects can be observed more than 2 wk after cessation of treatment (77). Although 17β-estradiol concentrations circulating in the blood may decline quite rapidly after removal of a sc silicone tubing implant containing 17β-estradiol (92), the behavioral response persists for at least a day (94), as does 17β-estradiol remaining bound in cells (105).

Intracranial and iv administration. Sex steroid hormones are extensively metabolized by the liver. To bypass this metabolism or the delay in delivery of the hormone to the neural site of action, hormones can be infused by cannula directly into the cerebral ventricles (106). If the neuroanatomical site of action of the hormones is being investigated, the hormone can be implanted directly into specific neuroanatomical areas (107). Similarly, both 17β-estradiol (96, 108) and progesterone (109–111) have been administered iv either to increase the amount of unmetabolized hormone reaching the brain or to deliver the hormone to the brain as rapidly as possible. In some cases, these modes of administration have been used when the particular compound being administered is costly (*e.g.* radioactively labeled estrogen or a difficult-to-isolate metabolite) or when a route that optimizes efficient delivery is desirable.

Pulses of hormones and sex differences. The most compelling evidence that different types of hormone replacement can produce different results comes from studies of sex differences in hormone-induced feminine sexual behavior in rats and guinea pigs. It is well known and accepted that there is a dramatic sex difference in response of many species to hormonal induction of feminine sexual behavior; after ovarian hormone treatment, the vast majority of gonadectomized female rats (112, 113) or guinea pigs (14, 114) express sexual behaviors, whereas most males do not (for review see Ref. 115). In contrast, if low-dose pulses of free 17β-estradiol are injected in an episodic manner separated by about 12–24 h and then are followed by progesterone injection, male rats (116, 117) and guinea pigs (118) express high rates of lordosis, sometimes as much as females. Although the reason that the pulses eliminate a quite robust sex difference seen with the more-typical single injections remains a fascinating mystery, it exemplifies the importance of considering the many options of hormonal replacement regimen for a particular study. The finding that pulse administration eliminates or attenuates the sex difference in sexual behavior should not be interpreted as a nuisance or an artifact; rather it tells us that the male brain interprets episodic 17β-estradiol exposure as meaning something different from continuous 17β-estradiol exposure. Because the brain of a female does not respond in a qualitatively different manner to episodic *vs.* continuous 17β-estradiol exposure, this represent an intriguing sex difference in response to different modes of hormone administration.

Experiments with mice. Mice are not just small rats, and their neuroendocrine systems are regulated in a number of ways that are different from rats. In particular, the hormonal regulation of sexual behaviors is different in mice than in rats, and even strains of mice can differ markedly from each other. This is made more difficult for the laboratory scientist by the relative paucity of information about hormones and brain function and behavior in mice. Researchers working in rats or guinea pigs, on the other hand, have a voluminous literature on the effects of different doses, injection regimens, routes of administration, and particular hormones to draw on. A good deal of this work on rats and guinea pigs came originally from the work of William C. Young's research group in the 1930s (80) (see *e.g.* Ref.119). This work has been added to and clarified many times over during the past half-century. This is not the case with mouse reproductive neuroendocrinology. Many of the critical parametric experiments have yet to be performed in mice. The result is that mice are used as a model species with which to study molecular neuroendocrine relationships involved in sexual behavior and other aspects of reproductive neuroendocrinology; however, unlike the situation in guinea pigs (120) and rats (85), less is known about the factors necessary for the induction of feminine sexual behavior during the estrous cycle. For example, unlike rats and guinea pigs, when female mice are given typical injections of 17β-estradiol followed by progesterone (106, 121, 122), or when given implants of a silicone tubing capsule of 17β-estradiol followed by pretest injections of progesterone (95), they do not show full sexual response until approximately the fourth weekly behavioral test. Furthermore, chronic estradiol treatment is not well tolerated by male mice (123, 124).

Complication of dosage in sex difference experiments. A complication of hormone dose arises when contrasts are made between males and females. When comparing the response of the two sexes to a particular hormonal treatment, should an equivalent amount of hormone be administered per animal or per body weight? Typically, for a particular age, male rats will be heavier than females, so providing a constant dose would be less of a dose to the males per body weight. Unfortunately, an informed decision about how to equate a dose requires a great deal of knowledge about the pharmacokinetics for a given hormone in the two sexes of a particular species and the amount of hormone present in the circulation after a particular injection (125, 126). These are empirical questions that will depend very much on the particular experiments and are well beyond the scope of this paper. Ideally, doses of a hormone would be administered to males and females that result in comparable concentrations of hormone in the circulation. If hyperphysiological, supersaturating doses of steroid hormones are administered, the differences in hormone concentrations may be inconsequential. However, a dose that produces physiological concentrations in females does not necessarily deliver an equivalent concentration in males. The pharmacokinetics of hormone and drug

disposition affects not only the peak of the hormone delivered but also the time course of hormone present in the blood system. So, a dose that produces equal concentrations of hormones in males and females at 30 min will probably not result in equal concentrations of hormones in blood at 2 h. Of course, all drugs and hormones are different, so each must be evaluated independently.

Summary. In summary, this section discussed the most common modes of administration and regimens used in ovarian hormone replacement treatment of rats. Each has its advantages and disadvantages, and each can be applied to the study of sex differences. A great deal of thought must go into choosing the particular hormones administered, their form, and the mode and timing of administration, and a good deal of thought must go into providing equitable treatments in males and females. Fortunately, much is already known about the effects of varying particular parameters on physiological responses, so well-informed choices are possible. Although testicular hormone replacement was not specifically discussed, all of the same considerations must be made in administering androgens as in administering estrogens and progestins.

Question 7: how do you study the stage of the menstrual cycle and other hormone variation in men and women?

Menstrual cycle. Just as is the case in laboratory animals, hormonal changes associated with a woman's reproductive cycle may contribute to sex differences observed. For example, the hormonal changes occurring during the normal menstrual cycle (*i.e.* from the follicular phase to the luteal phase; Fig. 2A) must be taken into account in evaluating many physiological or brain-based sex differences. The idea that men are hormonally static is not really true. Men, too, experience biological rhythms in the production of sex steroids, including significant diurnal and seasonal changes in testosterone production (30). A researcher studying sex differences should be aware of variations in endogenous hormones that may be pertinent to variables under study and be prepared to monitor them when appropriate. Thus, menstrual cycle stage in female subjects may need to be determined. Other major changes in sex steroids that could have significant implications for sex differences occur at puberty in both sexes, during pregnancy and the postpartum in women, and with aging.

During the typical menstrual cycle, serum concentrations of 17β-estradiol range from 30–300 pg/ml (127, 128), and peak concentrations are achieved directly before ovulation. It is important to realize that there can be large inter- and intra-individual variations in the exact timing of menstrual events and the concentrations of hormones attained at the various stages. Researchers should not assume that the length of a woman's cycle is 28 da In fact, the *median* length of the menstrual cycle is 29.5 d, and it is common for women to have regular cycles as short as 25 or as long as 35 d (127). Because the length of the luteal phase is relatively fixed at approximately 13–15 d, variations in cycle length largely reflect variation in the length of the follicular phase, and the timing of ovulation will vary accordingly. Concentrations of progesterone vary from less than 1.0 to around 15 ng/ml during the normal menstrual cycle, and peak progesterone concentrations are reached in the mid-luteal phase (127, 128). Follicular-phase progesterone concentrations are extremely low, along with concentrations in anovulatory and menopausal women. The late luteal phase is associated with a reduction in serum concentrations of 17β-estradiol, progesterone, or 17β-estradiol and progesterone. FSH concentrations range from less than 1.0 to 12 mIU/ml, whereas LH concentrations range from less than 2.0 to around 50 mIU/ml and peak at the time of ovulation (see Fig. 2A). Menstruation onset can be determined by simply asking women themselves or by analyzing hormone concentrations and comparing them with a chart similar to Fig. 2A.

Analyzing vaginal secretions (for day of maximum cervical mucus), analyzing urine samples (for daily estrone conjugates revealing ovarian follicular dynamics), taking basal body temperature, and using ovulation kits are all methods that can provide information regarding the approximate timing of ovulation (129, 130). Not all methods may be equally useful in a given study. For example, the rise in body temperature is small (between 0.2 and 0.5 C), occurs only after ovulation has transpired, and does not occur reliably in all women, even if cycles are ovulatory. Knowing the time of ovulation, however, can aid in the determination of the menstrual cycle stage of a female subject. To permit the proper adjustments to be made for menstrual cycle length, women should be asked to chart their cycle if there is doubt about its typical length. Monitoring two or more cycles may be needed to derive a reliable estimate (131). Although more elaborate methods are also available, this could be as simple as recording the date of menstrual onset over several cycles.

Puberty. The stages of puberty are conventionally defined in both sexes by the degree of development of secondary sex characteristics and rated on the Tanner scale (132). A complete description of the hormonal changes and stages of puberty in humans is beyond the scope of this article. Puberty is a time when females undergo major hormonal changes associated with the menstrual cycle (133). There is also a dramatic rise in androgen production in males.

Menopause. Menopause results from a sequence of events that leads to a modification in the programming along the hypothalamic-pituitary-gonadal (HPG) axis. After the menopause, production of 17β-estradiol and estrone by the ovaries declines considerably. A relationship between declining ovarian hormone concentrations and the symptoms of menopause has been suggested. The reciprocity between gonadal hormones and the serotonergic system presents some promising clues into the mechanism that initiates the onset of menopausally related neuroregulatory changes (134, 135).

Effects of hormones in humans. As mentioned earlier, to observe the effect that gonadal hormones have on human subjects in a study, the gonads cannot ethically be removed as they can in animals. Hormone concentrations, therefore, must be manipulated (*i.e.* added or reduced). This can be accomplished by capitalizing upon endogenous biological rhythms in steroid production or, ideally, by adding or removing hormones experimentally. For instance, women taking exogenous go-

nadal steroids for contraceptive purposes (*e.g.* oral contraceptives and GnRH analogs) or for therapeutic purposes (*e.g.* HRT) to palliate menopausal symptoms such as hot flushes and vaginal dryness or mood symptoms or GnRH analogs to treat premenstrual dysphoric disorder can be studied. Opportunities to study men are less frequent but include medical interventions such as androgen blockade in prostate cancer or testosterone replacement to prevent bone loss in men with HIV.

As one example, oral contraceptives suppress ovulation while sustaining menstruation due to regular steroid withdrawal. Presently, all low-dose oral contraceptives contain ethinyl estradiol in 20-, 30-, or 35-μg doses (136). These formulations are low in estrogen compared with the oral contraceptives that were on the market 25 yr ago. Presently, contraceptives containing 75–100 μg of mestranol or ethinyl estradiol are no longer available. Oral contraceptives containing 50 μg of ethinyl estradiol are available but mostly reserved for therapeutic situations where a higher dose of estrogen is required. The estrogen formulation used most frequently for HRT is Premarin, which is composed of conjugated equine estrogens that are a mixture of estrone sulfate (50%), equilin sulfate (23%), 17α-dihydroequilin sulfate (13%), and a variety of other conjugated estrogens (136).

The intestine inadequately absorbs progesterone. Two methods have been employed to make it orally active. One is to micronize the preparation. Progesterone has also been altered at C-17 and C-6 to make the potent oral progestins megestrol acetate and medroxyprogesterone acetate (136).

GnRH analogs can be administered sc or nasally to induce anovulation in women. GnRH agonists suppress ovulation because they down-regulate GnRH receptors in the hypothalamus, leading to decreased FSH and LH release from the pituitary, resulting in decreased estrogen and progesterone concentrations (137). In comparison with placebo, GnRH analogs are more effective at reducing cyclical mood changes related to the menstrual cycle (138–140). Long-term use of GnRH agonists is restricted by the incidence of side effects that mimic menopause and elevated risk for hypoestrogenism and osteoporosis. Evidence suggests that add-back therapy may help avoid some of these side effects (141). Intranasal buserelin at 400–900 μg daily or im leuprolide at 3.75–7.5 mg per month are the most suitable GnRH treatments for use clinically. Add-back therapy should be composed of conjugated estrogen at 0.625 mg daily (Monday to Saturday) and 10 mg of medroxyprogesterone acetate daily for 10 d during every fourth menstrual cycle (137).

Question 8: what are the effects of sex differences in pharmacokinetics and pharmacodynamics on the effects drug treatment?

Sex-related differences in drug effects. When measuring the response of males and females to drugs, it is important to remember that there may be pronounced sex differences. These arise from differences in pharmacodynamics (pharmacological effect on the body) and pharmacokinetics (absorption, distribution, metabolism, and excretion, or ADME) (for a review see Ref. 142). Pharmacodynamic differences have been reported for a number of drug classes, including analgesics, antidepressants, antipsychotics, and muscle relaxants. The underlying mechanisms, such as hormone-drug signaling interactions, are poorly understood and therefore not readily generalized. A careful survey of the literature is needed for each drug under study; moreover, one needs to recognize that sex difference may be species dependent. On the other hand, factors contributing to pharmacokinetic sex differences have been extensively documented for many species and provide a framework for anticipating differences in drug response between males and females. Sexually dimorphic metabolism of drugs and xenobiotics in the liver has been extensively documented (142). Physiological differences, summarized in Table 2, can cause higher plasma concentrations and longer half-lives of certain medications in women compared with men, including antidepressants. The tendency of women to have elevated plasma concentrations and longer half-lives of drugs may result in a higher incidence of side effects in women compared with men (143); however, plasma half-lives may also be shorter in females compared with males (142). The pharmacokinetics of certain drugs may also be altered by age. For example, younger women with depression are more responsive to serotonergic antidepressants (144). Similarly, changes do occur in males, for example with the circadian rhythm and with age, but potentially with different rates and amplitudes than in females.

Menstrual cycle. Hormonal fluctuations can have a strong effect on drug response in females. The endogenous hormonal changes that occur across the menstrual cycle have been associated with physiological changes that may affect pharmacokinetics in women. For example, compared with the follicular phase, the late luteal or premenstrual phase of the menstrual cycle is correlated with reduced gastric acid secretion as well as slower gastric emptying and short intestinal transit times (145). Other potential effects consist of increases in total body water, hepatic metabolism, creatinine clearance, and sodium retention (145).

A premenstrual reduction in drug concentrations is the main effect of these alterations (145). For instance, reductions in steady-state concentrations of desipramine and trazodone by as much as 53% during the late luteal phase have been reported (146). This may lead to the observance of sex differences in drug pharmacokinetics during the luteal phase but not the follicular phase, as has been reported for the

TABLE 2. Physiological differences in women that may affect drug pharmacokinetics

Physiological difference
Higher percentage of body fat
Higher cerebral blood flow
Slower gastric emptying time
Decreased gastric acid secretion
Lower body weight
Less blood volume
Lower plasma protein binding
Lower hepatic biotransformation
Slower renal clearance

[Adapted with permission from: S. G. Kornstein and C. K. Kirkwood: Handbook of Female Psychopharmacology (edited by M. Steiner and G. Koren), Martin Dunitz, London, 2003, p 1 (143).]

kinetics of lithium. There is also evidence that monoamine oxidase activity is reduced by estrogens and elevated by progesterone, suggesting that hormonal fluctuations (endogenous as well as exogenous) may affect mood in an unpredictable manner (147, 148).

Numerous studies have addressed the question how hormones affect drug metabolism and more specifically the oxidative cytochrome P450 enzymes, such as CYP3A4 (the main drug metabolizing CYP showing a tendency of greater activity in females), CYP2C9/19, CYP2D6, and CYP2E1. Although some studies have observed consistent changes as a function of hormonal effects, this depends on the CYP isozyme and the tissue analyzed. Overall, the changes do not appear to be dramatic in humans. In contrast, there are male-specific CYP isozymes in rats (CYP3A2 and CYP2C11) that can be induced in female livers upon administration of androgens (142). Even the induction of certain CYP isozymes by xenobiotics can show strong sex differences. Oral contraceptives may increase the metabolism of drugs that undergo conjugation or glucuronidation and, hence, may affect the clearance of drugs that are metabolized by oxidation in female patients (143). For example, decreased clearance and a 63% increase in the absolute bioavailability of imipramine was reported to be associated with concomitant oral contraceptive use (149). Taken together, pharmacokinetics and pharmacodynamics display clear differences between males and females. The magnitude of such differences varies with the drug under study, the species, tissue, and experimental conditions.

Pregnancy. During pregnancy, plasma concentrations of the acute-phase protein α1-acid glycoprotein vary dramatically (150). Because α1-acid glycoprotein represents a major drug-binding component in plasma, particularly under stimulated conditions, this affects the disposition of numerous drugs by enhancing plasma protein binding and affecting drug disposition.

There are also physiological changes of pregnancy that affect drug metabolism, including slowed gastric emptying, reduced gastrointestinal motility, increased volume of distribution, reduced protein binding capacity, and increased hepatic metabolism, which can substantially affect pharmacokinetic parameters that may lead to observed sex differences (151). For instance, in a study of the dose requirements for tricyclic antidepressants to ameliorate depressive symptoms during pregnancy, rising doses were required in the second half of pregnancy, with rapid dose escalation in the third trimester. The ultimate dose attained was about 1.6 times the prepregnancy dose (152).

Menopause. Menopausal status may also affect pharmacokinetic parameters, although it is sometimes difficult to separate the effects of menopausal status from the effects of age. Generally, one would expect reduced clearance, extended half-lives, and elevated plasma concentrations of medications (143). Exogenous hormone use, including HRT in postmenopausal women, oral contraceptives, and GnRH analogs may have confounding effects on the pharmacokinetics of other drugs that are administered. Conjugated HRT decreases (liquids) and increases (solids) gastric emptying times, reduces gastric acid secretion, and has variable effects on protein binding (145).

Question 9: when doing research on sex differences in humans, is it really necessary to measure hormone concentrations?

It depends on the research question being asked. If the organizational effects of sex steroids are being studied, measuring hormones is usually not feasible. This is because the behavior of interest is normally studied in childhood or adulthood, whereas the active hormones were present early in development, often prenatally. There is no practical way to measure hormone concentrations in the fetus. A few studies have tried measuring testosterone or other hormones in human amniotic fluid (*e.g.* (153), but because the amniocentesis procedure is performed at a fixed time in development, this method can at best provide a glimpse of individual differences in hormone concentrations, based on a single specimen, at one fixed time point. Whether this time point is important for any aspect of neuronal differentiation, and what behavioral systems might go through a critical period at that time, are unknown. There has been speculation (154, 155) that some aspects of sexual differentiation may occur in the first 6 months of postnatal life. If the speculations turn out to be true, it will open the door to researchers using direct measures of infants' hormones to study organizational effects. It may then be possible to link concentrations of specific hormones directly to patterns of behavioral development.

In situations where organizational effects are studied using clinical conditions, such as CAH, which was discussed above, it is still a technical problem to access fetal hormones. In addition, many of these conditions are not diagnosed until after birth. This means the hormonal abnormality is not recognized during the time when the central nervous system effects are presumably occurring. But in CAH, direct measures of hormones are a normal part of the postnatal diagnostic work-up. Serum assays of 17-hydroxyprogesterone and androgens, such as androstenedione or testosterone, may be useful during the period from birth to 2 wk after birth, or the point when treatment is usually initiated. Ongoing monitoring of hormones by a pediatric endocrinologist in children with CAH is used to help establish whether the patient's hormones are under satisfactory drug control. The clinical records can be useful to researchers for establishing that any androgen excess is limited to the period before treatment is initiated.

Direct vs. indirect manipulations of hormones. In most research situations where sex differences are studied, direct measures of hormone concentrations are desirable and often essential. The fact that the current hormone environment can modify the size of behavioral sex differences means that it is almost always good practice to measure the concentrations of relevant hormones. Even in situations where a dynamic effect is not suspected, it is worthwhile to measure hormones to demonstrate that such an effect is not present. But more often, researchers might find themselves in situations where a sex difference may need to be assessed at two or more concentrations of a given hormone. For instance, if there is a menstrual cycle effect on a particular behavioral or physical

variable, one might wish to assess the variable at high and low concentrations of 17β-estradiol or progesterone. In this situation, confirmation of the 17β-estradiol or progesterone concentrations would be required to demonstrate that the hormones varied as expected. In addition to changes in ovarian hormones over the menstrual cycle, a variety of other endogenous variation in steroid hormone concentrations can be used to manipulate hormones. For example, there are diurnal changes in testosterone and cortisol release (32, 156). However, it must be kept in mind that these manipulations are indirect methods of studying the effects of a particular hormone. Because there is variation across individuals in the timing and concentrations of hormones attained, actual measurement of hormone concentrations is usually essential to verify that the expected changes in hormones are present and therefore that the manipulation is valid. For instance, on average, men's free testosterone concentrations decline by 50% between ages 30 and 80 (31). However, there are some 80-yr-olds who have higher testosterone than some 35-yr-olds. Therefore, the only way to conclusively demonstrate that testosterone is lower in the older group is to measure it directly.

It is particularly important to include direct measurement of ovarian hormones when doing menstrual cycle studies. Historically, this has not always been done. There are a few situations where the timing of the cycle itself is of interest, such as in studies of premenstrual syndrome or in studies where disruptions in the length or regularity of the cycle are significant endpoints. More typically, the goal of the work is to draw inferences about the actions of reproductive hormones. In this situation, tracking the phases of the cycle is insufficient. Phases of the menstrual cycle in women are defined either by their timing (*e.g.* the mid-luteal phase occurs about 1 wk before the onset of menstruation) or by associated events (*e.g.* the presence of menses), not by the exact concentrations of hormones achieved. Therefore, if the purpose of a study is to make inferences about a particular hormone, there is no substitute for directly measuring the hormone itself. This is especially true because there are large individual differences in the precise concentrations of hormones attained at each phase of the cycle and in the timing of menstrual cycle events across individual women and across multiple cycles within an individual woman. Conversely, it is not possible to tell whether a woman is in the middle of the luteal phase, based strictly on the concentrations of 17β-estradiol and progesterone that are present. What constitutes a mid-luteal value in one woman may only be an early luteal phase value in another. Large parts of the cycle cannot be confirmed through hormonal measurements alone because of the individual differences in hormone concentrations attained and can only be verified retrospectively based on the onset of the next menstrual period or by following changes in hormones over a number of menstrual cycles.

Another method for studying the effects of hormones on brain function is to manipulate hormone concentrations directly. In humans, options for doing this are limited by ethical considerations. As noted earlier, effects of HRT in older women have been studied as a way of experimentally manipulating estrogen concentrations. Less attention has been paid to the progestin component of HRT. The effects of testosterone replacement in older men have also been studied in a few investigations (*e.g.* Ref. 157). Randomized controlled trials are the best method for studying the effects of a hormonal manipulation. However, because randomized controlled trials are typically short-term, they do not mimic the natural conditions under which many people use hormones and cannot detect cumulative long-term effects of the hormone treatments. Therefore, studies of long-term users and nonusers also have an important role to play. In this type of study, however, matching on a large range of relevant health and demographic variables is of the utmost importance. In studies where a hormone is directly administered or suppressed, hormone assays still have an important role to play. For instance, there may be questions of compliance that the assays can address, or questions related to how individual differences in metabolism relate to outcomes. Researchers must ensure the target hormones of interest are accurately reflected in their assay. Not all synthetic hormones can be detected using standard assay procedures (*e.g.* ethinyl estradiol, the estrogen used in most oral contraceptives, has low cross-reactivity with the antisera used in conventional RIA kits, and, therefore, special assays must be performed to detect it).

Serum vs. saliva assays. Finally, is serum or saliva the better medium for quantifying hormone concentrations? Measuring hormones in serum is still the most widely used method for measuring hormone concentrations in both research and clinical settings. Direct assays are available for a wide range of peptide and steroid hormones, including the full range of reproductive hormones relevant to sex difference studies. The assays are well validated, widely available, and can be performed by most commercial laboratories using isotopic or nonisotopic techniques.

Saliva-based assays are a recent innovation and are widely used in Europe, at least for some hormones (*e.g.* cortisol). Saliva-based assays have been slower to catch on in North America but offer some important theoretical advantages. In particular, most assays of serum hormone concentrations measure the total hormone in blood, whereas saliva-based assays measure the unbound, or more accurately the bioavailable fraction, of a steroid hormone (158). Saliva-based assays, therefore, provide a useful index of precisely that fraction of the hormone that exerts biological effects (see Table 3 and Figs. 5 and 6). This derives from the fact that large peptides cannot diffuse from blood into the saliva, so steroids bound to binding globulins will not be found in saliva. On the downside, it also means that a smaller spectrum of hormones is represented in saliva compared with serum (*e.g.* LH and FSH are not found in saliva to any significant extent). The ease and cost-effectiveness of saliva collection, long shelf-life at ambient temperatures, and noninvasiveness of the procedures offer significant advantages in some research settings. For instance, endocrine studies involving children, repeated specimen collection over minutes, days, or weeks, and field studies that involve specimen collection outside the laboratory are more feasible. Technical obstacles associated with the detection of lower hormone concentrations have been the challenge for developers of saliva-based assays and explain

TABLE 3. Hormone levels: comparison of seruma and saliva concentrations

Hormone	No. of specimens	R value	Citation
17-OH progesterone (total serum *vs.* saliva)	13	0.98	(196)
Estriol (unconjugated)	24	0.97	(197)
Cortisol (free)	93	0.97	(193)
Human chorionic gonadotropin	24	0.56	(198)
Progesterone (total serum *vs.* saliva)	96	0.88	(199)
Estradiol (total serum *vs.* saliva)	14	0.82	(200)
Cortisol (total serum *vs.* saliva)	13	0.93	Diagnostic Systems Laboratories, Inc. (Webster, TX)

[Adapted with permission from L. Hofman: J Nutr 131:16215, 2001 (165). © American Society for Nutritional Sciences.]

the slow adoption of this promising technology. Because only the free or bioavailable fraction is contained in saliva, the concentrations are often near or below the lower detection threshold of standard immunoassays. Nevertheless, techniques are under development and rapidly gaining acceptance. Although still not as widely available as serum assays, mature assays are available in saliva for cortisol (Fig. 5), progesterone, and testosterone (in men; see also Fig. 6) (159, 160). 17β-Estradiol remains technically difficult to measure in saliva, and working assays are generally available only through a few research labs at present. The measurement of testosterone in women is still controversial in both serum and saliva. A recent review showed that in serum, only two of 10 commercially available immunoassays gave an accurate measurement of testosterone in women, judging by concurrent gas chromatography-mass spectrometry on the same specimens (161). Saliva kits were not assessed.

Researchers interested in using saliva-based assays must carefully research their assay and collection procedures. The ease of collection disguises the many pitfalls awaiting the unwary investigator. For instance, use of cotton as a collection device is fine for cortisol but produces inaccurate readings for many other steroids (162). Steroids in saliva are less vulnerable to enzymatic breakdown than in serum but are still subject to bacterial degradation of the specimens. Amending specimens with sodium azide to reduce bacterial activity is often recommended but can interfere with some types of immunoassays. Care must also be taken in the choice of a saliva stimulant, use of plastic *vs.* glass for storage, and when to prepare the mouth for specimen collection. A useful review of some of the critical variables can be found in Ref. 163 or 164. For a general introduction to saliva hormone monitoring, see Ref. 165 or 166.

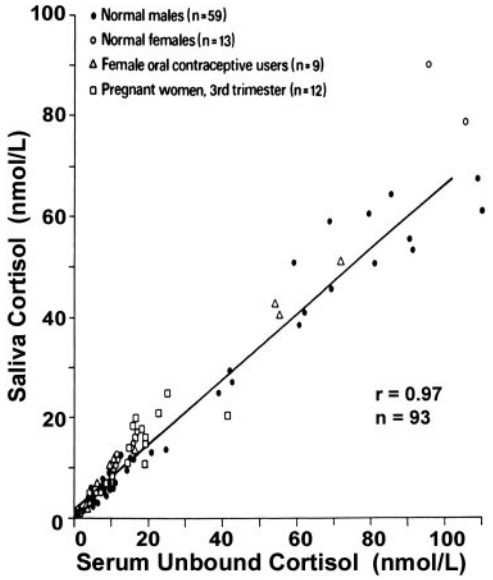

FIG. 5. Cortisol in saliva closely reflects the bioavailable fraction of the hormone in serum. The bioavailable component of a steroid hormone is often the component of interest in research studies, and saliva has desirable qualities as a medium for quantifying steroid hormones. Shown here are correlations between saliva and serum measures cortisol in men and women. Saliva assay techniques are less well developed than for serum. Therefore, researchers who elect to use saliva must take care to choose an assay technique that is well validated to ensure accurate results. [Adapted with permission from R. Vining *et al.*: Ann Clin Biochem 20:329, 1983 (193). © Association of Clinical Biochemists.]

Question 10: how does stress impact the study of sex differences?

Although many describe the HPG axis as a simple closed biological system, there are other factors that can influence hormone secretion patterns and, thus, sex differences. Immediately relevant to the study of sex differences is the effect of stress and stress hormones (*e.g.* catecholamines and glucocorticoids) on both male and female physiology and behavior. Animal and human studies indicate that gonadal steroid secretion is markedly altered by stress and glucocorticoids in a sex- and hormone-dependent fashion. In addition, sex steroids themselves can differentially affect stress responsiveness and can thus result in differential respon-

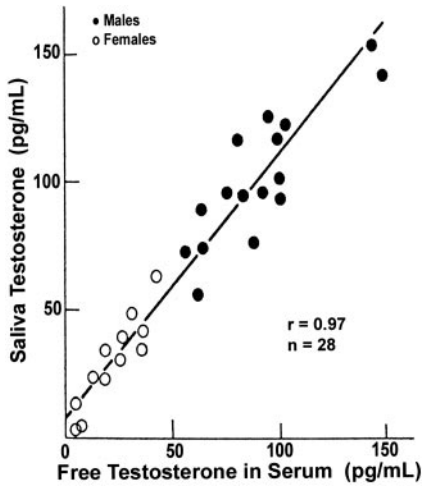

FIG. 6. Correlation of unbound serum and salivary testosterone concentrations. [Adapted with permission from J. Vittek: Life Sci 37:711, 1985 (194). © Elsevier, Inc.]

siveness to incidental stress in males *vs.* females or gonadally manipulated animals on behavioral or pharmacological measures (Fig. 7). Thus, it is important to consider the potential mitigating influence of stress and stress hormones when planning experiments.

Laboratory animals. The negative impact of stress on the rodent HPG axis has been documented in numerous studies (see Refs. 167–170 for review). As depicted in Fig. 7, acute stress can inhibit GnRH and LH secretion. This can happen within minutes, likely through direct actions of CRH on GnRH neurons (171). Chronic stress disrupts the HPG axis at multiple levels; chronic stress reduces release of GnRH at the median eminence, decreases pituitary sensitivity to GnRH, and directly interferes with LH stimulation of sex-hormone-producing cells (see Ref. 172). Stress inhibition of the HPG system occurs in both female and male rats (see (173). Physical stressors such as infection also appear to involve glucocorticoids as part of the mechanism involved in their suppression of the HPG axis (174).

It is also important to note that in some systems, stress may have opposite effects in males and female (175, 176). Thus, incidental, noncontrolled stress exposure has the potential to produce effects that are associated with sex differences in the stress response, rather than sex differences in the particular parameter being explored.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis produces both between-sex and within-sex differences in activation that can play a role in behavior and physiology.

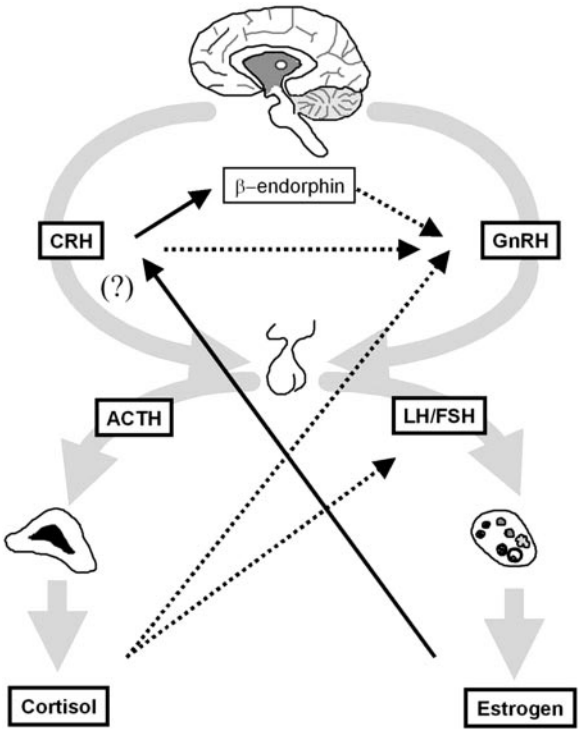

FIG. 7. Interactions between the HPA and the hypothalamic-pituitary-ovarian axes (*solid lines* and *broken lines* represent stimulatory and inhibitory actions, respectively; the *question mark* reflects uncertainty regarding the locus of central estrogen action on the HPA axis). [Reproduced with permission from E. Young and A. Korszun: Psychiatr Clin North Am 21:309, 1998 (195). © Elsevier, Inc.]

For example, in rats, both basal and stress-induced corticosterone secretion is more pronounced in females than males (177, 178). Whereas basal hypersecretion may be in part accounted for by elevated corticosteroid-binding globulin in females (178), corticosterone responses to stress remain exaggerated, reflecting a greater net impact on glucocorticoid secretion. In addition, HPA secretion profiles vary across the estrous cycle in the rat; proestrous or estrous females show elevated basal and stress-induced corticosterone levels relative to diestrous animals (177, 178), consistent with a hypothesized HPA-facilitatory action of estrogens. Thus, the influence of cyclically secreted or administered gonadal steroids may cause a disproportionate impact on female rats exposed to incidental stress exposure.

It is important to consider the manipulation of the male when conducting sex differences studies. Surgery is known to produce lasting effects on the HPA axis (179, 180), and thus it is critical that sex differences studies involving gonadectomy employ appropriate operated control groups. In addition, vaginal smears likely produce some degree of a stress response in female rats; although there is no equivalent in the male, it is important that males have parallel handling to account for this potential difference in stress history.

Finally, the impact of the glucocorticoids on the HPG axis may vary with the time of day. The HPA axis shows a marked circadian rhythm, with corticosteroids peaking at the onset of the active phase of an animal's or human's light-dark cycle (see Ref. 156). The change in concentrations of corticosterone from nadir to peak can exceed 40-fold, with binding of cognate receptors shifting from minimal to extensive (181). Correspondingly, the impact of glucocorticoids on behavioral and physiological sex differences may depend on the time of testing within the animal's light-dark cycle. Overall, glucocorticoid fluxes associated with stress or the circadian cycle can clearly inhibit the HPG axis and need to be taken into account in sex difference studies in rodents.

Humans. In humans, studies suggest that stress is also inhibitory to the reproductive axis. However, because we cannot intentionally subject women to long-term stress, the evidence is more circumstantial. The classic example of stress-induced inhibition of the reproductive axis is hypothalamic amenorrhea, also known as stress-induced amenorrhea. In this disorder, slowing of LH pulses in the presence of low 17β-estradiol and progesterone suggest a central mediation of the effect of stress acting directly on the LH pulse generator (182). Most disorders of reproductive functioning in humans (*e.g.* hypothalamic amenorrhea, exercise-induced amenorrhea, and anorexia nervosa) are accompanied by activation of the HPA axis (183–185). However, the link is not definitive, and data to date do not suggest marked reproductive abnormalities in major depression, despite documented activation of the HPA axis (186, 187). Although most of the nonhuman primate data have concentrated on CRH as the primary mediator of this inhibition, there are some data suggesting that glucocorticoids may also be an important mediator. Cushing's syndrome, a disorder of extremely high cortisol production caused by either a pituitary or adrenal adenoma, is accompanied by inhibition of the reproductive

axis and infertility. Diminished LH response to GnRH after long-term prednisolone treatment has been found in women (188). Stress also results in inhibition of testosterone secretion in men, secondary to inhibition of the central HPG axis (189).

As in rodent models, time of day of assessment of any end point is an important consideration. The peak of the HPA axis in humans occurs around awakening with the nadir around bedtime or shortly after falling asleep. In humans, there is strong circadian entrainment in the reproductive axis during puberty, when LH secretion occurs predominantly at night. In sexually mature humans, there is still some circadian rhythm in reproductive hormones that can be altered by disease states so the circadian rhythm should be considered in reproductive hormone assessment (190).

Summary. In light of the potential confounding effects of stress on measures of sex differences in brain and behavior, it is highly recommended that studies of sex differences be designed with the possible impact of stress in mind. Specifically, 1) efforts should be taken to limit extraneous sensory stimuli in animal holding rooms or experimental environments; 2) if procedures must involve handling or restraint (*e.g.* injections), subjects should be well habituated to the procedure before the actual testing session to obviate possible effects of an acute stress response; and 3) time of day needs to be considered in all experimental designs. If possible, the time of testing should be held constant across conditions, to eliminate the possibility that diurnal glucocorticoid changes can modulate gonadal steroid-regulated processes.

Closing comments

Studies that are carefully designed to test a clear hypothesis regarding sex differences and the effects of gonadal steroids are crucial to achieving two distinct, though related, aims. One is to determine whether a sex or gender difference has significant clinical relevance in humans and should, therefore, be considered in developing and using preventive, diagnostic, or therapeutic interventions. The other is to further our understanding of human and animal biology, to answer the question, "What does this difference tell us about the underlying biology of this system?"

The importance of sex differences has long been recognized by neuroscientists, beginning with early descriptions of sexual dimorphisms in behavior and neuroanatomy (7, 14, 191, 192). Significant advances in the technology available for studying the nervous system are now coupled with a more complete understanding of the interconnectedness of the nervous and endocrine systems. A thorough understanding of the capabilities and challenges of research methods in this area is a necessary prerequisite to designing studies that take advantage of current knowledge and thinking in this area.

For those who work at, or consider entering, the intersection of endocrinology and neuroscience, the choices of experimental models, designs, and methodologies may seem daunting. It is hoped that this paper provides useful guidance in addressing these choices, and the authors welcome readers' comments and additions to this work.

Glossary

Androgens. A class of sex steroid hormones associated with the development and maintenance of the secondary male sex characteristics, sperm induction, and sexual differentiation.

Aromatase. An enzyme that converts testosterone to 17β-estradiol.

Assay. The determination of the amount of a particular constituent of a mixture or of the biological or pharmacological potency of a drug.

Basal body temperature. The temperature taken at its lowest point in the day, usually in the morning before getting out of bed.

Classic endocrine experiment. Endocrine gland removal followed by hormone replacement therapy.

Congenital adrenal hyperplasia (CAH). A genetic disorder present at birth characterized by a deficiency of the hormones aldosterone and cortisol and an overproduction of androgens.

Conjugated equine estrogens. Thought to be the active ingredients in Premarin; conjugated refers to the fact that the estrogens are chemically bonded to a sulfate group, and equine refers to the fact that the hormones are isolated from urine collected from pregnant horses.

Corpus luteum. A yellow glandular mass in the ovary formed by an ovarian follicle that has matured and discharged its ovum. The corpus luteum secretes progesterone.

Defeminization. Any change that renders an animal less like typical females.

Demasculinization. Any change that renders an animal less like typical males.

Endogenous. Developing or originating within the organism or arising from causes within the organism.

Estradiol. 17β-Estradiol is a sex steroid hormone synthesized mainly in the ovary, but also in the placenta, testis, and adrenal cortex.

Estriol. A metabolite of 17β-estradiol and usually the predominant estrogenic metabolite in urine. During pregnancy, large amounts of estriol are produced by the placenta.

Estrogens. A class of sex steroid hormones associated with expression of secondary female sex characteristics, predominantly synthesized by the ovary.

Estrone. A metabolite of 17β-estradiol but possessing less biological activity. It is found in the urine of pregnant women and mares. It is also the dominant hormone found in postmenopausal women.

Estrous. Adjective that refers to the female reproductive cycle or the day of behavioral estrus when used to modify a noun such as estrous cycle or estrous female.

Estrus. Noun that refers to that portion or phase of the reproductive cycle of female animals characterized by ovulation and willingness to permit coitus.

Fadrozole. A selective aromatase inhibitor.

Feminine. Describes traits that are typical of females.

Feminization. A change that makes an animal more like typical females (not the same as demasculinization).

Flutamide. An androgen receptor antagonist.

Follicular phase. The follicular phase, also referred to as the proliferative phase, is the preovulatory phase of a woman's reproductive cycle during which the follicle grows and high serum estrogen concentrations cause the uterine lining to grow.

Glucocorticoids. A class of adrenal steroid hormones that affect carbohydrate metabolism (gluconeogenesis, liver glycogen deposition, and elevation of blood sugar), inhibit corticotropin secretion, and possess

pronounced antiinflammatory activity. Glucocorticoids can inhibit gonadal secretions.

Gonadotropin-releasing hormone (GnRH). A peptide hormone that is produced and released by the hypothalamus and regulates the production and release of gonadotropins from the pituitary gland.

Hormone replacement therapy (HRT). Also referred to as hormone treatment, or HT. Administration of hormones, commonly used to refer to treatment with estrogens and often progestins after menopause.

Lordosis. The position that estrous females of many species exhibit when a male mounts. The position is characterized by dorsiflexion of the lumbar curvature of the spine.

Luteal phase. The postovulatory phase of the female reproductive cycle, also referred to as the secretory phase.

Masculine. Describes traits that are typical of males.

Masculinization. A change that makes an animal more like typical males (not the same as defeminization).

Müllerian-inhibiting hormone (substance). A glycoprotein secreted by the embryonic testis that causes involution of the Müllerian duct systems.

Progesterone. A progestin synthesized mainly in the ovary.

RU486. A progestin and glucocorticoid receptor antagonist.

Sexual dimorphism. The differences between female and male individuals of a species.

SRY. A gene found on the Y chromosome that determines the formation of testes.

Tanner scale. A standardized method for describing the stage of pubertal development in humans that uses parameters of physical development that requires assessment of secondary sex characteristics.

Testosterone. Sex steroid hormone, of the class of androgens, secreted by gonads and adrenals.

Acknowledgments

We thank Viviana Simon for her help with the manuscript and for formatting the figures. We thank Michael Baum for his helpful comments on an earlier version of the manuscript.

Received August 27, 2004. Accepted November 10, 2004.

Address all correspondence and requests for reprints to: Jill B. Becker University of Michigan, 525 East University, Ann Arbor, Michigan 48109. E-mail: jbbecker@umich.edu.

This work was supported by an unrestricted educational grant from Ortho-McNeil Pharmaceuticals to the Society for Women's Health Research.

References

1. **Wizemann TM, Pardue ML** 2001 Exploring the biological contributions to human health: does sex matter? Washington, DC: Board on Health Sciences Policy, Institute of Medicine
2. **Whalen RE** 1982 Current issues in the neurobiology of sexual differentiation. In: Vernadakis A, Timaras PS, eds. Hormones in development and aging. New York: Spectrum Publications; 273–304
3. **Arnold AP** 2002 Concepts of genetic and hormonal induction of vertebrate sexual differentiation in the twentieth century, with special reference to the brain. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Rubin R, eds. Hormones, brain, and behavior. San Diego: Academic Press
4. **Goodfellow PN, Lovell-Badge R** 1993 SRY and sex determination in mammals. Annu Rev Genet 27:71–92
5. **Jost A** 1970 Hormonal factors in the sex differentiation of the mammalian foetus. Philos Trans R Soc Lond B Biol Sci 259:119–130
6. **Nef S, Parada LF** 2000 Hormones in male sexual development. Genes Dev 14:3075–3086
7. **Goy RW, McEwen BS** 1980 Sexual differentiation of the brain. Cambridge, MA: MIT Press
8. **MacLusky NJ, Naftolin F** 1981 Sexual differentiation of the central nervous system. Science 211:1294–1302
9. **Arnold AP, Gorski RA** 1984 Gonadal steroid induction of structural sex differences in the central nervous system. Annu Rev Neurosci 7:413–442
10. **Breedlove S, Hampson E** 2002 Sexual differentiation of brain and behavior. In: Becker J, Breedlove S, Crews D, McCarthy M, eds. Behavioral endocrinology. 2nd ed. Cambridge, MA: MIT Press/Bradford Books; 75–114
11. **Moore C, Power K** 1992 Variation in maternal care and individual differences in play, exploration, and grooming of juvenile Norway rat offspring. Dev Psychobiol 25:165–182
12. **Moore CL** 1982 Maternal behavior of rats is affected by hormonal condition of pups. J Comp Physiol Psychol 96:123–129
13. **Moore CL** 1986 A hormonal basis for sex differences in the self-grooming of rats. Horm Behav 20:155–165
14. **Phoenix CH, Goy RW, Gerall AA, Young WC** 1959 Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65:369–382
15. **Arnold AP, Breedlove SM** 1985 Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm Behav 19:469–498
16. **Arnold AP, Agate RJ, Carruth LL** 2003 Hormonal and non-hormonal mechanisms of sexual differentiation of the brain. In: Legato M, ed. Principles of gender-specific medicine. San Diego: Elsevier
17. **Becker J, Robinson T, Lorenz K** 1982 Sex differences and estrous cycle variations in amphetamine-elicited rotational behavior. Eur J Pharmacol 80: 65–72
18. **McClintock M** 1971 Menstrual synchrony and suppression. Nature 229:244–245
19. **McClintock M** 1998 On the nature of mammalian and human pheromones. Ann NY Acad Sci 855:390–392
20. **McClintock M** 1984 Estrous synchrony: modulation of ovarian cycle length by female pheromones. Physiol Behav 32:701–705
21. **Camp DM, Becker JB, Robinson TE** 1986 Sex differences in the effects of gonadectomy on amphetamine-induced rotational behavior in rats. Behav Neural Biol 46:491–495
22. **De Vries GJ** 2004 Sex differences in adult and developing brains: compensation, compensation, compensation. Endocrinology 145:1063–1068
23. **Bradshaw W, Baum M, Awh C** 1981 Attenuation of 5α-reductase inhibitor of the activational effects of testosterone propionate on penile erections in castrated male rats. Endocrinology 109:1047–1051
24. **Breedlove SM, Arnold AP** 1983 Hormonal control of a developing neuromuscular system. I. Complete demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide. J Neurosci 3:417–423
25. **Etgen AM** 1979 Antiestrogens: effects of tamoxifen, nafoxidine, and CI-628 on sexual behavior, cytoplasmic receptors, and nuclear binding of estrogen. Horm Behav 13:97–112
26. **Brown TJ, Blaustein JD** 1984 Inhibition of sexual behavior in female guinea pigs by a progestin receptor antagonist. Brain Res 301:343–349
27. **Brown TJ, Moore MJ, Blaustein JD** 1987 Maintenance of progesterone-facilitated sexual behavior in female rats requires continued hypothalamic protein synthesis and nuclear progestin receptor occupation. Endocrinology 121:298–304
28. **Azcoitia I, Sierra A, Veiga S, Honda S, Harada N, Garcia-Segura LM** 2001 Brain aromatase is neuroprotective. J Neurobiol 47:318–329
29. **Korach KS** 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266:1524–1527
30. **Hampson E** 2002 Sex differences in human brain and cognition: the influence of sex steroids in early and adult life. In: Becker JB, Breedlove SN, Crews D, McCarthy MM, eds. Behavioral endocrinology. 2nd ed. Cambridge, MA: MIT Press/Bradford Books; 579–628
31. **Vermeulen A, Kaufman JM** 1995 Ageing of the hypothalamo-pituitary-testicular axis in men. Horm Res 43:25–28
32. **Nieschlag E** 1974 Circadian rhythm of plasma testosterone. In: Aschoff J, Ceresa F, Halberg F, eds. Chronobiological aspects of endocrinology. Stuttgart, Germany: Schattauer Verlag; pp 117–128.
33. **Smals AG, Kloppenborg PW, Benraad TJ** 1976 Circannual cycle in plasma testosterone levels in man. J Clin Endocrinol Metab 42:979–982
34. **Sherwin BB** 1988 Estrogen and/or androgen replacement therapy and cognitive functioning in surgically menopausal women. Psychoneuroendocrinology 13:345–357
35. **Salminen E, Portin R, Korpela J, Backman H, Parvinen LM, Helenius H, Nurmi M** 2003 Androgen deprivation and cognition in prostate cancer. Br J Cancer 89:971–976
36. **Duff SJ, Hampson E** 2000 A beneficial effect of estrogen on working memory in postmenopausal women taking hormone replacement therapy. Horm Behav 38:262–276
37. **Shaywitz SE, Shaywitz BA, Pugh KR, Fulbright RK, Skudlarski P, Mencl WE, Constable RT, Naftolin F, Palter SF, Marchione KE, Katz L, Shankweiler DP, Fletcher JM, Lacadie C, Keltz M, Gore JC** 1999 Effect of estrogen on brain activation patterns in postmenopausal women during working memory tasks. J Am Med Assoc 281:1197–1202
38. **Olster DH, Blaustein JD** 1989 Development of steroid-induced lordosis in female guinea pigs: effects of different estradiol and progesterone treatments, clonidine, and early weaning. Horm Behav 23:118–129

39. **Breedlove S, Arnold A** 1983 Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus. J Neurosci 3:424–432
40. **Nordeen EJ, Nordeen KW, Sengelaub DR, Arnold AP** 1985 Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229:671–673
41. **De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-Badge R, Burgoyne PS, Arnold AP** 2002 A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. J Neurosci 22:9005–9014
42. **Fitch RH, Denenberg VH** 1998 A role for ovarian hormones in sexual differentiation of the brain. Behav Brain Sci 21:311–327; discussion 327–352
43. **Berenbaum SA, Duck SC, Bryk K** 2000 Behavioral effects of prenatal *vs.* postnatal androgen excess in children with 21-hydroxylase-deficient congenital adrenal hyperplasia. J Clin Endocrinol Metab 85:727–733
44. **Zucker KJ, Bradley SJ, Oliver G, Blake J, Fleming S, Hood J** 1996 Psychosexual development of women with congenital adrenal hyperplasia. Horm Behav 30:300–318
45. **Romeo RD, Richardson HN, Sisk CL** 2002 Puberty and the maturation of the male brain and sexual behavior: recasting a behavioral potential. Neurosci Biobehav Rev 26:381–391
46. **Nunez JL, Sodhi J, Juraska JM** 2002 Ovarian hormones after postnatal day 20 reduce neuron number in the rat primary visual cortex. J Neurobiol 52:312–321
47. **Cline TW, Meyer BJ** 1996 Vive la difference: males vs females in flies vs worms. Annu Rev Genet 30:637–702
48. **Capel B** 1998 Sex in the 90s: SRY and the switch to the male pathway. Annu Rev Physiol 60:497–523
49. **Burgoyne PS** 1993 A Y-chromosomal effect on blastocyst cell number in mice. Development 117:341–345
50. **Renfree MB, Short RV** 1988 Sex determination in marsupials: evidence for a marsupial-eutherian dichotomy. Philos Trans R Soc Lond B Biol Sci 322: 41–53
51. **Reisert I, Pilgrim C** 1991 Sexual differentiation of monoaminergic neurons: genetic or epigenetic? Trends Neurosci 14:468–473
52. **Carruth LL, Reisert I, Arnold AP** 2002 Sex chromosome genes directly affect brain sexual differentiation. Nat Neurosci 5:933–934
53. **Dewing P, Shi T, Horvath S, Vilain E** 2003 Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. Mol Brain Res 118:82–90
54. **Mahadevaiah SK, Odorisio T, Elliott DJ, Rattigan A, Szot M, Laval SH, Washburn LL, McCarrey JR, Cattanach BM, Lovell-Badge R, Burgoyne PSl** 1998 Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities. Hum Mol Genet 7:715–727
55. **Lovell-Badge R, Robertson E** 1990 XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. Development 109: 635–646
56. **Arnold A, Burgoyne P** 2004 Are XX and XY brain cells intrinsically different? Trends Endocrinol Metab15:6–11
57. **Markham JA, Jurgens HA, Auger CJ, De Vries GJ, Arnold AP, Juraska JM** 2003 Sex differences in mouse cortical thickness are independent of the complement of sex chromosomes. Neuroscience 116:71–75
58. **Parker KL, Schedl A, Schimmer BP** 1999 Gene interactions in gonadal development. Annu Rev Physiol 61:417–433
59. **Eckel L, Houpt T, Geary N** 2000 Spontaneous meal patterns in female rats with and without access to running wheels. Physiol Behav 70:397–405
60. **Freeman ME** 1994 The neuroendocrine control of the ovarian cycle of the rat. In: Knobil E, Neill J, eds. The physiology of reproduction. 2nd ed. New York: Raven Press; 613–658
61. **Long J, Evans H** 1922 The oestrous cycle in the rat and its associated phenomena. In: Leuschner A, ed. Memoirs of the University of California. Berkeley, CA: University of California Press; 1–149
62. **Blandau R, Boling J, Young W** 1941 The length of heat in the albino rat as determined by the copulatory response. Anat Rec 79:453–463
63. **McCarthy MM, Becker JB** 2002 Neuroendocrinology of sexual behavior in the female. In: Becker JB, Breedlove SM, Crews D, McCarthy MM, eds. Behavioral endocrinology. 2nd ed. Cambridge, MA: MIT Press/Bradford Books; 117–151
64. **Lucas P, Donohoe S, Thody A** 1982 The role of estrogen and progesterone in the control of preputial gland sex attractant odors in the female rat. Physiol Behav 28:601–607
65. **Freeman M, Smith M, Nazian S, Neill J** 1974 Ovarian and hypothalamic control of the daily surges of prolactin secretion during pseudopregnancy in the rat. Endocrinology 94:875–882
66. **Freeman M, Butcher R, Fugo N** 1970 Alterations of oocytes and follicles by delayed ovulation. Biol Reprod 2:209–215
67. **Feder HH** 1981 Estrous cyclicity in mammals. In: Adler NT, ed. Neuroendocrinology of reproduction: physiology and behavior. New York: Plenum Press; 279–348
68. **Frick K, Berger-Sweeney J** 2001 Spatial reference memory and neocortical neurochemistry vary with the estrous cycle in C57BL/6 mice. Behav Neurosci 115:229–237
69. **Snell GD** 1941 Biology of the laboratory mouse. Philadelphia: The Blakiston Co.
70. **Allen E** 1922 The oestrous cycle of the mouse. Am J Anat 30:297–371
71. **Katzenellenbogen BS** 1984 Biology and receptor interactions of estriol and estriol derivatives in vitro and in vivo. J Steroid Biochem 20:1033–1037
72. **Moss RL, McCann SM** 1973 Induction of mating behavior in rats by luteinizing hormone-releasing factor. Science 181:177–179
73. **Smith ER, Damassa DA, Davidson JM** 1977 Hormone administration: peripheral and intracranial implants. In: Myers RD, ed. Methods in psychobiology. New York: Academic Press; 259–278
74. **Johnston PG, Davidson JM** 1979 Priming action of estrogen: minimum duration of exposure for feedback and behavioral effects. Neuroendocrinology 28:155–159
75. **Martucci CP, Fishman J** 1979 Impact of continuously administered catechol estrogens on uterine growth and luteinizing hormone secretion. Endocrinology 105:1288–1292
76. **Jezierski MK, Sohrabji F** 2001 Neurotrophin expression in the reproductively senescent forebrain is refractory to estrogen stimulation. Neurobiol Aging 22:309–319
77. **Patisaul HB, Aultman EA, Bielsky IF, Young LJ, Wilson ME** 2003 Immediate and residual effects of tamoxifen and ethynylestradiol in the female rat hypothalamus. Brain Res 978:185–193
78. **Garcia-Segura LM, Baetens D, Naftolin F** 1986 Synaptic remodelling in arcuate nucleus after injection of estradiol valerate in adult female rats. Brain Res 366:131–136
79. **Hlinak Z** 1993 Social recognition in ovariectomized and estradiol-treated female rats. Horm Behav 27:159–166
80. **Young WC** 1969 Psychobiology of sexual behavior in the guinea pig. In: Lehrman DS, Hinde RA, Shaw E, eds. Advances in the study of behavior. New York: Academic Press; 1–110
81. **Baum MJ** 2002 Neuroendocrinology of sexual behavior in the male. In: Becker JB, Breedlove SM, Crews D, McCarthy MM, eds. Behavioral endocrinology. Cambridge, MA: MIT Press/ Bradford Books; 153–203
82. **Sodersten P, Eneroth P, Hansen S** 1981 Induction of sexual receptivity in ovariectomized rats by pulse administration of oestradiol-17β. J Endocrinol 89:55–62
83. **Clark AS, Roy EJ** 1987 Effective intervals for the administration of estradiol pulses and the induction of sexual behavior in female rats. Physiol Behav 39:665–667
84. **Davidson JM, Rodgers CH, Smith ER, Bloch GJ** 1968 Stimulation of female sex behavior in adrenalectomized rats with estrogen alone. Endocrinology 82:193–195
85. **Powers JB** 1970 Hormonal control of sexual receptivity during the estrous cycle of the rat. Physiol Behav 5:831–835
86. **Blaustein JD, Erskine MS** 2002 Feminine sexual behavior: cellular integration of hormonal and afferent information in the rodent forebrain. In: Pfaff DW, Arnold AP, Etgen AM, Fahrbach SE, Rubin RT, eds. Hormones, brain and behavior. New York: Academic Press; 139–214
87. **Leavitt WW, Bosley CG, Blaha GC** 1971 Source of ovarian preovulatory progesterone. Nat New Biol 234:283–284
88. **Asarian L, Geary N** 2002 Cyclic estradiol treatment normalizes body weight and restores physiological patterns of spontaneous feeding and sexual receptivity in ovariectomized rats. Horm Behav 42:461–471
89. **Dziuk PJ, Cook B** 1966 Passage of steroids through silicone rubber. Endocrinology 78:208–211
90. **Wise PM, Rance N, Barraclough CA** 1981 Effects of estradiol and progesterone on catecholamine turnover rates in discrete hypothalamic regions in ovariectomized rats. Endocrinology 108:2186–2193
91. **McGinnis MY, Krey LC, MacLusky NJ, McEwen BS** 1981 Steroid receptor levels in intact and ovariectomized estrogen-treated rats: an examination of quantitative, temporal and endocrine factors influencing the efficacy of an estradiol stimulus. Neuroendocrinology 33:158–165
92. **Legan SJ, Coon GA, Karsch FJ** 1975 Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 96:50–56
93. **Henderson SR, Baker C, Fink G** 1977 Oestradiol-17β and pituitary responsiveness to luteinizing hormone releasing factor in the rat: a study using rectangular pulses of estradiol-17β monitored by non-chromatographic radioimmunoassay. J Endocrinol 73:441–453
94. **Parsons B, McEwen BS, Pfaff DW** 1982 A discontinuous schedule of estradiol treatment is sufficient to activate progesterone-facilitated feminine sexual behavior and to increase cytosol receptors for progestins in the hypothalamus of the rat. Endocrinology 110:613–619
95. **Rissman EF, Early AH, Taylor JA, Korach KS, Lubahn DB** 1997 Estrogen receptors are essential for female sexual receptivity. Endocrinology 138:507–510
96. **Blaustein JD, Dudley SD, Gray JM, Roy EJ, Wade GN** 1979 Long-term retention of estradiol by brain cell nuclei and female rat sexual behavior. Brain Res 173:355–359
97. **Micevych P, Eckersell CB, Holland K, Smith A** 1996 Induction of CCK

mRNA levels in the limbic-hypothalamic circuit: time course and site-specific effects of estrogen. J Neurobiol 30:465–479
98. **Tapper CM, Greig F, Brown-Grant K** 1974 Effects of steroid hormones on gonadotrophin secretion in female rats after ovariectomy during the oestrous cycle. J Endocrinol 62:511–525
99. **Goodman RL** 1978 A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. Endocrinology 102:142–150
100. **Attardi B** 1981 Facilitation and inhibition of the estrogen-induced luteinizing hormone surge in the rat by progesterone: effects on cytoplasmic and nuclear estrogen receptors in the hypothalamus-preoptic area, pituitary, and uterus. Endocrinology 108:1487–1496
101. **Karsch FJ, Dierschke DK, Weick RF, Yamaji T, Hotchkiss J, Knobil E** 1973 Positive and negative feedback control by estrogen of luteinizing hormone secretion in the rhesus monkey. Endocrinology 92:799–804
102. **Schwartz SM, Blaustein JD, Wade GN** 1979 Inhibition of estrous behavior by progesterone in rats: role of neural estrogen and progestin receptors. Endocrinology 105:1078–1082
103. **Walker WA, Feder HH** 1977 Inhibitory and facilitatory effects of various anti-estrogens on the induction of female sexual behavior by estradiol benzoate in guinea pigs. Brain Res 134:455–465
104. **Blaustein JD, Feder HH** 1979 Cytoplasmic progestin-receptors in guinea pig brain: characteristics and relationship to the induction of sexual behavior. Brain Res 169:481–497
105. **Parsons B, MacLusky NJ, Krey L, Pfaff DW, McEwen BS** 1980 The temporal relationship between estrogen-inducible progestin receptors in the female rat brain and the time course of estrogen activation of mating behavior. Endocrinology 107:774–779
106. **Mani SK, Allen JM, Clark JH, Blaustein JD, O'Malley BW** 1994 Convergent pathways for steroid hormone- and neurotransmitter-induced rat sexual behavior. Science 265:1246–1249
107. **Rubin BS, Barfield RJ** 1983 Induction of estrous behavior in ovariectomized rats by sequential replacement of estrogen and progesterone to the ventromedial hypothalamus. Neuroendocrinology 37:218–224
108. **Green R, Luttge WG, Whalen RE** 1970 Induction of receptivity in ovariectomized female rats by a single intravenous injection of estradiol-17. Physiol Behav 5:137–141
109. **Meyerson B** 1972 Latency between intravenous injection of progestins and the appearance of estrous behavior in estrogen-treated ovariectomized rats. Horm Behav 3:1–9
110. **McGinnis MY, Parsons B, Rainbow TC, Krey LC, McEwen BS** 1981 Temporal relationship between cell nuclear progestin receptor levels and sexual receptivity following intravenous progesterone administration. Brain Res 218:365–371
111. **Glaser JH, Rubin BS, Barfield RJ** 1983 Onset of the receptive and proceptive components of feminine sexual behavior in rats following the intravenous administration of progesterone. Horm Behav 17:18–27
112. **Beach FA** 1938 Sex reversals in the mating pattern of the rat. J Genet Psychol 53:225–262
113. **Ball J** 1939 Male and female mating behavior in prepubertally castrated male rats receiving estrogens. J Comp Psychol 28:273–283
114. **Grady KL, Phoenix CH, Young WC** 1965 Role of the developing rat testis in differentiation of the neural tissues mediating mating behavior. J Comp Physiol Psychol 59:176–182
115. **Baum MJ** 1979 Differentiation of coital behavior in mammals: a comparative analysis. Neurosci Biobehav Rev 3:265–284
116. **Sodersten P, Pettersson A, Eneroth P** 1983 Pulse administration of estradiol-17β cancels sex difference in behavioral estrogen sensitivity. Endocrinology 112:1883–1885
117. **Olster DH, Blaustein JD** 1988 Progesterone facilitation of lordosis in male and female Sprague-Dawley rats following priming with estradiol pulses. Horm Behav 22:294–304
118. **Olster DH, Blaustein JD** 1990 Biochemical and immunocytochemical assessment of neural progestin receptors following estradiol treatments that eliminate the sex difference in progesterone-facilitated lordosis in guinea-pigs. J Neuroendocrinol 2:79–86
119. **Boling JL, Blandau RJ** 1939 The estrogen-progesterone induction of mating responses in the spayed female rat. Endocrinology 25:359–364
120. **Joslyn WD, Feder HH, Goy RW** 1971 Estrogen conditioning and progesterone facilitation of lordosis in guinea pigs. Physiol Behav 7:477–482
121. **Edwards DA** 1970 Induction of estrus in female mice: estrogen-progesterone interactions. Horm Behav 1:299–304
122. **Thompson ML, Edwards DA** 1971 Experiential and strain determinants of the estrogen-progesterone induction of sexual receptivity in spayed female mice. Horm Behav 2:299–305
123. **Streng T, Li X, Lehtoranta M, Makela S, Poutanen M, Talo A, Tekmal RR, Santti R** 2002 Infravesical obstruction in aromatase over expressing transgenic male mice with increased ratio of serum estrogen-to-androgen concentration. J Urol 168:298–302
124. **Li X, Makela S, Streng T, Santti R, Poutanen M** 2003 Phenotype characteristics of transgenic male mice expressing human aromatase under ubiquitin C promoter. J Steroid Biochem Mol Biol 86:469–476
125. **Kato R** 1974 Sex-related differences in drug metabolism. Drug Metab Rev 3:1–32
126. **Conney AH** 1967 Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev 19:317–366
127. **Chabbert Buffet N, Djakoure C, Maitre SC, Bouchard P** 1998 Regulation of the human menstrual cycle. Front Neuroendocrinol 19:151–186
128. **Thorneycroft I, Mishell DJ, Stone S, Kharma K, Nakamura R** 1971 The relation of serum 17-hydroxyprogesterone and estradiol-17-β levels during the human menstrual cycle. Am J Obstet Gynecol 111:947–951
129. **Fehring RJ** 2002 Accuracy of the peak day of cervical mucus as a biological marker of fertility. Contraception 66:231–235
130. **Lasley BL, Shideler SE, Munro CJ** 1991 A prototype for ovulation detection: pros and cons. Am J Obstet Gynecol 165:2003–2007
131. **Freeman EW, DeRubeis RJ, Rickels K** 1996 Reliability and validity of a daily diary for premenstrual syndrome. Psychiatry Res 65:97–106
132. **Tanner J** 1962 Growth and adolescence. Oxford, UK: Blackwell
133. **Angold A, Costello EJ, Worthman CM** 1998 Puberty and depression: the roles of age, pubertal status and pubertal timing. Psychol Med 28:51–61
134. **Fink G** 1995 The psychoprotective action of estrogen is mediated by central serotonergic as well as dopaninergic mechanisms. In: Takada A, Curzon G, eds. Serotonin in the central nervous system and periphery. Amsterdam: Elsevier Science; 175–187
135. **Fink G, Sumner BE, Rosie R, Grace O, Quinn JP** 1996 Estrogen control of central neurotransmission: effect on mood, mental state, and memory. Cell Mol Neurobiol 16:325–344
136. **PDR Staff** 2004 Physicians' desk reference. 58th ed. New York: Thomson Healthcare
137. **Steiner M, Yonkers KA** 2003 Evidence-based treatment of mood disorders in women. Ment Fit 2:34–67
138. **Bancroft J, Boyle H, Warner P, Fraser HM** 1987 The use of an LHRH agonist, buserelin, in the long-term management of premenstrual syndromes. Clin Endocrinol (Oxf) 27:171–182
139. **Muse KN, Cetel NS, Futterman LA, Yen SC** 1984 The premenstrual syndrome: effects of "medical ovariectomy". N Engl J Med 311:1345–1349
140. **Schmidt PJ, Nieman LK, Danaceau MA, Adams LF, Rubinow DR** 1998 Differential behavioral effects of gonadal steroids in women with and in those without premenstrual syndrome. N Engl J Med 338:209–216
141. **Mortola JF, Girton L, Fischer U** 1991 Successful treatment of severe premenstrual syndrome by combined use of gonadotropin-releasing hormone agonist and estrogen/progestin. J Clin Endocrinol Metab 72:252A–252F
142. **Gandhi M, Aweeka F, Greenblatt R, Blaschke T** 2004 Sex differences in pharmacokinetics and pharmacodynamics. Annu Rev Pharmacol Toxicol 44:499–523
143. **Kornstein SG, Kirkwood CK** 2003 Antidepressant drugs. In: Steiner M, Koren G, eds. Handbook of female psychopharmacology. London: Martin Dunitz; 1–18
144. **Grigoriadis S Kennedy SH Bagby RM** 2003 A comparison of antidepressant response in younger and older women. J Clin Psychopharmacol 23:405–407
145. **Yonkers KA, Brawman-Mintzer O** 2002 The pharmacologic treatment of depression: is gender a critical factor? J Clin Psychiatry 63:610–615
146. **Kimmel S, Gonsalves L, Youngs Dea** 1992 Fluctuating levels of antidepressants premenstrually. J. Psychosom Obstet Gynecol 13:277–280
147. **Klaiber EL, Kobayashi Y, Broverman DM, Hall F** 1971 Plasma monoamine oxidase activity in regularly menstruating women and in amenorrheic women receiving cyclic treatment with estrogens and a progestin. J Clin Endocrinol Metab 33:630–638
148. **Klaiber EL, Broverman DM, Vogel W, Peterson LG, Snyder MB** 1996 Individual differences in changes in mood and platelet monoamine oxidase (MAO) activity during hormonal replacement therapy in menopausal women. Psychoneuroendocrinology 21:575–592
149. **Abernethy DR, Greenblatt DJ, Shader RI** 1984 Imipramine disposition in users of oral contraceptive steroids. Clin Pharmacol Ther 35:792–797
150. **Havenaar EC, Axford JS, Brinkman-van der Linden EC, Alavi A, Van Ommen EC, van het Hof B, Spector T, Mackiewicz A, Van Dijk W** 1998 Severe rheumatoid arthritis prohibits the pregnancy-induced decrease in α3-fucosylation of α1-acid glycoprotein. Glycoconj J 15:723–729
151. **Stowe ZN, Strader JR, Nemeroff CB** 1998 Psychopharmacology during pregnancy and lactation. In: Schatzberg AF, Nemeroff CB, eds. Textbook of psychopharmacology. 2nd ed. Washington, DC: American Psychiatric Press; 979–996
152. **Wisner KL, Perel JM, Wheeler SB** 1993 Tricyclic dose requirements across pregnancy. Am J Psychiatry 150:1541–1542
153. **Finegan JA, Bartleman B, Wong PY** 1989 A window for the study of prenatal sex hormone influences on postnatal development. J Genet Psychol 150:101–112
154. **Hampson E** 1995 Spatial cognition in humans: possible modulation by androgens and estrogens. J Psychiatry Neurosci 20:397–404
155. **Collaer ML, Hines M** 1995 Human behavioral sex differences: a role for gonadal hormones during early development? Psychol Bull 118:55–107
156. **Keller-Wood ME, Dallman MF** 1984 Corticosteroid inhibition of ACTH secretion. Endocr Rev 5:1–24

157. **Janowsky JS, Oviatt SK, Orwoll ES** 1994 Testosterone influences spatial cognition in older men. Behav Neurosci 108:325–332
158. **Quissell DO** 1993 Steroid hormone analysis in human saliva. Ann NY Acad Sci 694:143–145
159. **Read GF** 1993 Status report on measurement of salivary estrogens and androgens. Ann NY Acad Sci 694:146–160
160. **Ellison PT** 1993 Measurements of salivary progesterone. Ann NY Acad Sci 694:161–176
161. **Taieb J, Mathian B, Millot F, Patricot MC, Mathieu E, Queyrel N, Lacroix I, Somma-Delpero C, Boudou P** 2003 Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography-mass spectrometry in sera from 116 men, women, and children. Clin Chem 49:1381–1395
162. **Shirtcliff EA, Granger DA, Schwartz E, Curran MJ** 2001 Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. Psychoneuroendocrinology 26:165–173
163. **Ellison PT** 1988 Human salivary steroids: Methodological considerations and applications in physical anthropology. Yearbook Phys Anthropol 31:115–142
164. **Groschl M, Wagner R, Rauh M, Dorr HG** 2001 Stability of salivary steroids: the influences of storage, food and dental care. Steroids 66:737–741
165. **Hofman L** 2001 Human saliva as a diagnostic specimen. J Nutr 131:1621S–1625S
166. **Vining R, McGinley R** 1986 Hormones in saliva. Crit Rev Clin Lab Sci 23:95–146
167. **Kitay JI** 1961 Sex differences in adrenal cortical secretion in the rat. Endocrinology 68:818–824
168. **Karsch FJ, Battaglia DF, Breen KM, Debus N, Harris TG** 2002 Mechanisms for ovarian cycle disruption by immune/inflammatory stress. Stress 5:101–112
169. **Dobson H, Ghuman S, Prabhakar S, Smith R** 2003 A conceptual model of the influence of stress on female reproduction. Reproduction 125:151–163
170. **Calogero AE, Bagdy G, D'Agata R** 1998 Mechanisms of stress on reproduction: evidence for a complex intra-hypothalamic circuit. Ann NY Acad Sci 851:364–370
171. **Gambacciani M, Yen SS, Rasmussen DD** 1986 GnRH release from the mediobasal hypothalamus: in vitro inhibition by corticotropin-releasing factor. Neuroendocrinology 43:533–536
172. **Rivier C, Rivest S** 1991 Effect of stress on the activity of the hypothalamic-pituitary-gonadal axis: peripheral and central mechanisms. Biol Reprod 45: 523–532
173. **Bidzinska B, Petraglia F, Angioni S, Genazzani AD, Criscuolo M, Ficarra G, Gallinelli A, Trentini GP, Genazzani AR** 1993 Effect of different chronic intermittent stressors and acetyl-l-carnitine on hypothalamic β-endorphin and GnRH and on plasma testosterone levels in male rats. Neuroendocrinology 57:985–990
174. **Debus N, Breen KM, Barrell GK, Billings HJ, Brown M, Young EA, Karsch FJ** 2002 Does cortisol mediate endotoxin-induced inhibition of pulsatile luteinizing hormone and gonadotropin-releasing hormone secretion? Endocrinology 143:3748–3758
175. **Shors TJ, Chua C, Falduto J** 2001 Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus. J Neurosci 21:6292–6297
176. **Wood GE, Beylin AV, Shors TJ** 2001 The contribution of adrenal and reproductive hormones to the opposing effects of stress on trace conditioning in males versus females. Behav Neurosci 115:175–187
177. **Viau V, Meaney MJ** 1991 Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 129: 2503–2511
178. **Carey MP, Deterd CH, de Koning J, Helmerhorst F, de Kloet ER** 1995 The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. J Endocrinol 144:311–321
179. **Schmidt ED, Binnekade R, Janszen AW, Tilders FJ** 1996 Short stressor induced long-lasting increases of vasopressin stores in hypothalamic corticotropin-releasing hormone (CRH) neurons in adult rats. J Neuroendocrinol 8:703–712
180. **Prewitt CM, Herman JP** 1997 Hypothalamo-pituitary-adrenocortical regulation following lesions of the central nucleus of the amygdala. Stress 1:263–280
181. **Reul JM, de Kloet ER** 1985 Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505–2511
182. **Reame NE, Sauder SE, Case GD, Kelch RP, Marshall JC** 1985 Pulsatile gonadotropin secretion in women with hypothalamic amenorrhea: evidence that reduced frequency of gonadotropin-releasing hormone secretion is the mechanism of persistent anovulation. J Clin Endocrinol Metab 61:851–858
183. **Suh BY, Liu JH, Berga SL, Quigley ME, Laughlin GA, Yen SS** 1988 Hypercortisolism in patients with functional hypothalamic-amenorrhea. J Clin Endocrinol Metab 66:733–739
184. **Berga SL, Mortola JF, Girton L, Suh B, Laughlin G, Pham P, Yen SS** 1989 Neuroendocrine aberrations in women with functional hypothalamic amenorrhea. J Clin Endocrinol Metab 68:301–308
185. **Loucks AB, Mortola JF, Girton L, Yen SS** 1989 Alterations in the hypothalamic-pituitary-ovarian and the hypothalamic-pituitary-adrenal axes in athletic women. J Clin Endocrinol Metab 68:402–411
186. **Young EA, Carlson NE, Brown MB** 2001 Twenty-four-hour ACTH and cortisol pulsatility in depressed women. Neuropsychopharmacology 25:267–276
187. **Young EA, Midgley AR, Carlson NE, Brown MB** 2000 Alteration in the hypothalamic-pituitary-ovarian axis in depressed women. Arch Gen Psychiatry 57:1157–1162
188. **Sakakura M, Takebe K, Nakagawa S** 1975 Inhibition of luteinizing hormone secretion induced by synthetic LRH by long-term treatment with glucocorticoids in human subjects. J Clin Endocrinol Metab 40:774–779
189. **Morgan 3rd CA, Wang S, Mason J, Southwick SM, Fox P, Hazlett G, Charney DS, Greenfield G** 2000 Hormone profiles in humans experiencing military survival training. Biol Psychiatry 47:891–901
190. **Jaffe RB, Plosker S, Marshall L, Martin MC** 1990 Neuromodulatory regulation of gonadotropin-releasing hormone pulsatile discharge in women. Am J Obstet Gynecol 163:1727–1731
191. **Arnold A** 2004 Sex chromosomes and brain gender. Nat Rev Neurosci 5:701–705
192. **Berkley K, Hoffman G, Murphy A, Holdcroft A** 2002 Pain: sex/gender differences. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Rubin R, eds. Hormones, brain and behavior. New York: Academic Press; 409–442
193. **Vining R, McGinley R, Maksvytis J** 1983 Salivary cortisol: a better measure of adrenal cortical function than serum cortisol. Ann Clin Biochem 20:329–335
194. **Vittek J** 1985 Direct RIA of salivary testosterone. Life Sci 37:711–716
195. **Young E, Korszun A** 1998 Psychoneuroendocrinology of depression: hypothalamic-pituitary-gonadal axis. Psychiatr Clin North Am 21:309–323
196. **Price D, Astin M, Chard C, Addison G** 1979 Assay of hydroxyprogesterone in saliva. Lancet 2:368–369
197. **Vining R, McGinley R, Rice B** 1983 Saliva estriol measurements: an alternative to the assay of serum unconjugated estriol in assessing fetoplacental function. J Clin Endocrinol Metab 56:454–460
198. **Vining R, McGinley R, Simons R** 1983 Hormones in saliva: mode of entry and consequent implications for clinical interpretation. Clin Chem 29.
199. **DeBoever J, Kohen F, Vanderkerckhove D** 1986 Direct solid-phase chemiluminescence immunoassay for salivary progesterone. Clin Chem 32:763–767
200. **Worthman C, Stallings J, Hofman L** 1990 Sensitive salivary estradiol assay for monitoring ovarian function. Clin Chem 36:1769–1773
201. **Smith MS, Freeman ME, Neill JD** 1975 The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96:219–226

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**