

Exhibit D

Scientific Statement

Considering Sex as a Biological Variable in Basic and Clinical Studies: An Endocrine Society Scientific Statement

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Abbreviations: ACTH, adrenocorticotrophic hormone; AT₂R, angiotensin type 2 receptor; BMI, body mass index; cAMP, cyclic adenosine monophosphate; CKD, chronic kidney disease; CRF, corticotropin-releasing factor; CVD, cardiovascular disease; dMRI, diffusion magnetic resonance imaging; fMRI, functional magnetic resonance imaging; FCG, Four Core Genotypes (model); GMV, gray matter volume; GPCR, G-protein coupled receptor; HPA, hypothalamic-pituitary-adrenal; KYN, kynurenine; LC, locus coeruleus; MIH, Müllerian inhibitory hormone; PAR, pseudoautosomal region; PKA, protein kinase A; PTSD, posttraumatic stress disorder; RAAS, renin-angiotensin-aldosterone system; rs-fMRI, resting state functional magnetic resonance imaging; sMRI, structural magnetic resonance imaging; UCN, urocortin.

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Abstract

In May 2014, the National Institutes of Health (NIH) stated its intent to “require applicants to consider sex as a biological variable (SABV) in the design and analysis of NIH-funded research involving animals and cells.” Since then, proposed research plans that include animals routinely state that both sexes/genders will be used; however, in many instances, researchers and reviewers are at a loss about the issue of sex differences. Moreover, the terms *sex* and *gender* are used interchangeably by many researchers,

further complicating the issue. In addition, the sex or gender of the researcher might influence study outcomes, especially those concerning behavioral studies, in both animals and humans. The act of observation may change the outcome (the “observer effect”) and any experimental manipulation, no matter how well-controlled, is subject to it. This is nowhere more applicable than in physiology and behavior. The sex of established cultured cell lines is another issue, in addition to aneuploidy; chromosomal numbers can change as cells are passaged. Additionally, culture medium contains steroids, growth hormone, and insulin that might influence expression of various genes. These issues often are not taken into account, determined, or even considered. Issues pertaining to the “sex” of cultured cells are beyond the scope of this Statement. However, we will discuss the factors that influence sex and gender in both basic research (that using animal models) and clinical research (that involving human subjects), as well as in some areas of science where sex differences are routinely studied. Sex differences in baseline physiology and associated mechanisms form the foundation for understanding sex differences in diseases pathology, treatments, and outcomes. The purpose of this Statement is to highlight lessons learned, caveats, and what to consider when evaluating data pertaining to sex differences, using 3 areas of research as examples; it is not intended to serve as a guideline for research design.

Key Words: brain-gut, cardiovascular disease, chromosome complement, gender, sex differences, steroid hormones

Sex is an important biological variable that must be considered in the design and analysis of human and animal research. The terms *sex* and *gender* should not be used interchangeably. Sex is dichotomous, with sex determination in the fertilized zygote stemming from unequal expression of sex chromosomal genes. By contrast, gender includes perception of the individual as male, female, or other, both by the individual and by society; both humans and animals have sex, but only humans have gender. Both sexes produce estrogens, androgens, and progestins; there are no male- or female-specific sex hormones, *per se*, although these steroids are present in substantially different levels in males and females. Sex differences are caused by 3 major factors—sex hormones, genes, and environment. To understand disease mechanisms and exploit sex differences in protection or exacerbation of diseases, one needs to determine the relative contribution of factors, including observer effect (1), causing sex differences. Here—using 3 broad research areas as examples—the roles of sex differences in brain anatomy, brain-gut axis, and cardiovascular disease are discussed. Contemporary brain imaging methods show age- and sex-related differences in brain size, global and regional gray matter volume, white matter connectivity, and neuroanatomic regulation of appetite and satiety; while these differences are seen in large population-based studies, there is tremendous individual overlap, but such group-level findings do not inform findings, physiology, or pathology at the individual level. Sex differences in disorders of the brain-gut axis, obesity, type 2 diabetes,

and metabolic syndrome are caused by differential actions of brain-gut peptide and steroid hormones. The activation, signaling, and pharmacotherapy responses of the components of the hypothalamic-pituitary-adrenal (HPA) axis differ between the sexes. Heart and kidney functions are linked. Age, hormones, and sex biases seen in cardiovascular and chronic kidney diseases also differentially influence pharmacologic responses in men and women. Thus, sex differences pervade biology and medicine, and while not discussed in this Statement, must be considered in virtually all areas of biomedical research.

Section I

Sex Versus Gender

Much of the American public is surprisingly prudish about the word *sex*; it has now become commonplace to use the seemingly more genteel term *gender* when one really means *sex*. In *Moritz v Commissioner of Internal Revenue* (469 F. 2d 466 [1972]), Ruth Bader Ginsburg (subsequently, The Honorable Ruth Bader Ginsburg) argued against discrimination “on the basis of sex” not “on the basis of gender,” thus clearly, knowledgeably, and presciently understanding that “sex” does not equal “gender.” In a decision 48 years later (*Bostock v Clayton County*, 590 US, decided June 15, 2020), the United States Supreme Court separately ruled against discrimination on the basis of gender. *Gender* is often misused as a synonym for *sex*—for example, when filling out forms for various activities, we are routinely

asked to check a box labeled “gender,” but the only available options are boxes labeled “M” and “F.” But *sex* is not the same thing as *gender* and using these terms as equivalents obfuscates differences that are real and important in society in general and biomedical research in particular.

Biological Sex: The Definition of Male and Female

Sex is a biological concept. Asexual reproduction (cloning) is routine in microorganisms and some plants, but most vertebrates and all mammals have 2 distinct sexes. Even single-cell organisms have “mating types” to facilitate sexual reproduction. Only cells belonging to different mating types can fuse together to reproduce sexually (2, 3). Sexual reproduction allows for exchange of genetic information and promotes genetic diversity. The classical biological definition of the 2 sexes is that females have ovaries and make larger female gametes (eggs), whereas males have testes and make smaller male gametes (sperm); the 2 gametes fertilize to form the zygote, which has the potential to become a new individual. The advantage of this simple definition is first that it can be applied universally to any species of sexually reproducing organism. Second, it is a bedrock concept of evolution, because selection of traits may differ in the 2 sexes. Thirdly, the definition can be extended to the ovaries and testes, and in this way the categories—female and male—can be applied also to individuals who have gonads but do not make gametes.

In mammals, numerous sexual traits (gonads, genitalia, etc) that typically differ in males and females are tightly linked to each other because one characteristic leads to sex differences in other traits. The type of gonads is controlled by the presence of XX or XY chromosomes, and gonadal secretions in turn regulate formation of female or male reproductive tissues, and characteristics that differ in typical males or females. These characteristics include external genitalia, uterus and oviducts, sperm ducts, and secondary sexual characteristics such as facial hair and pitch of voice. However, many people cannot make either eggs or sperm, yet are recognized as female or male based on other physical characteristics; people who do not have either ovaries or testes are rare. For individuals that possess a combination of male- and female-typical characteristics, these clusters of traits are sufficient to classify most individuals as either biologically male or female. For example, a person with testes and a penis, who cannot make sperm, is usually classified as a biological male, as long as the person does not possess female features such as a vagina, ovaries, or uterus. Based on evidence presented, to define male and female individuals in general society, we expand the defining characteristics of sex to include nongonadal traits, as well as classical gonadal traits.

A simple biological definition of male and female, satisfactory to all people, is elusive. In human societies, the terms *female* and *male* can have several meanings, as they refer both to a person’s biological sex and to their social roles. Most people learn to discriminate males and females from an early age, but often not based on biological traits (4). For example, behaviors such as pair-bonding, sexual activity, offspring defense and care, and mate/partner selection (5) involve complex interplay between sex steroid hormones and peptide hormones (oxytocin and arginine vasopressin); these behaviors are encouraged differently in women and men, which influences their role in the society and culture in which they live to behave as “females” or “males.” While these factors have little impact on their biological sex, they can have profoundly different outcomes in the behavior and health of an individual. Biological sex is dichotomous because of the different roles of each sex in reproduction. For scientific research, it is important to define biological sex and distinguish it from other meanings.

Sex Chromosomes and Biological Sex Determination

Among mammals and many other taxa, males are characterized as the heterogametic sex (6), having 2 different sex chromosomes, X and Y, whereas females are homogametic (XX). By contrast birds, many reptiles, and some other organisms have Z and W chromosomes (7). In these organisms, the female is the heterogametic sex (ZW) and males are homogametic (ZZ). Some adult fish and reptiles can also change sex in response to environmental factors (8, 9), and even the adult mouse gonad can undergo partial sex reversal when specific genes are deleted (10, 11). Human biological sex is often assessed by examining the individual’s complement of sex chromosomes as determined by karyotypic analysis: males are XY and females are XX. Karyotypic sex is actually a surrogate for genetic sex, determined by the presence of the *SRY* gene on the Y chromosome (12, 13). However, karyotypic analysis may be misleading, as there are well-described 46,XX males (with testes). Most of these individuals carry a short segment of the Y chromosome that includes *SRY* transferred to an X chromosome, but up to 10% lack an *SRY* gene (14, 15). Similarly, there are 46,XY females, who have *SRY* but also have a duplication of *DAX1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) (16).

Sex Determination and Sex Differentiation

In mammals, sex determination begins with the inheritance of XX or XY chromosomes, which are the only factors that are different in XX and XY zygotes. Thus, all phenotypic sex differences, including gonadal development, stem originally from the unequal effects of XX and XY

sex chromosomes. Phenotypic sex differences develop in XX and XY embryos as soon as transcription begins. The categories of X and Y genes that are unequally represented or expressed in male and female mammalian zygotes, which could cause phenotypic sex differences, fall into 3 main categories (17).

1. *Y genes causing male-specific effects.* These Y-linked genes do not have homologous genes on the X chromosome. The most important Y-linked gene is *SRY*, the testis-determining gene, which encodes the *SRY* transcription factor expressed during embryonic life in the bipotential gonadal ridge; *SRY* activates downstream autosomal genes such as *SOX9* to cause formation of a testis (18). In the absence of *SRY*, autosomal and X chromosome genes (*WNT-4*, *DAX-1*, *FOXL2*, *COUP-TFII*, and *RSPO1*) are activated to cause formation of an ovary (19-22). Both testicular and ovarian development are subject to active genetic regulation (12, 13, 16). Pathways downstream of *SRY* inhibit ovary-determining pathways, and ovary-determining pathways also inhibit pathways for testis development. Once the testes form, they secrete sex hormones that act widely throughout the body to cause male differentiation of nongonadal tissues. Other Y genes also have male-specific effects (for example, those required for spermatogenesis) (23, 24).
2. *X gene dosage or parental imprint.* Because XX nongermline cells inactivate one X chromosome (25, 26), it was long thought that both XX and XY cells have only one active X chromosome, with little inherent difference in expression related to the number of X chromosomes. The inactivated regions of the X chromosome are “coated” with large noncoding RNA transcribed from the X-inactive specific transcript (*XIST*) gene, part of the XIC (X inactivation center) located on Xq13 (27, 28). But some genes escape X inactivation (termed as *X escapees*), and therefore are expressed more in XX than XY cells, resulting in imbalance or incomplete dosage compensation (29). About 23% of human X-linked genes are more abundantly expressed in XX cells than XY cells in many tissues (30, 31). Recent evidence from mouse studies suggests that the inherent male-female difference in expression of X genes leads to significant sex differences in disease phenotypes. For example, sex differences in placental *Ogt* expression are associated with sex differences in prenatal vulnerability to stress (32). X escapee *Kdm6a*, a histone demethylase, contributes to sex differences in mouse models of bladder cancer (33), autoimmune disease (34), and Alzheimer disease (35). Similarly, variations in human *KDM6A* are associated with prognosis of bladder cancer or cognitive decline in female patients (33). The dose of another X escapee histone demethylase, *Kdm5c*,

contributes to sex differences in adiposity and body weight in mice, and variations in *KDM5C* in humans are associated with body mass (36).

Sex differences may also arise from genes in the pseudoautosomal regions (PARs) of the sex chromosomes, small regions of sequence similarity on the X and Y chromosomes that allow for X and Y chromosome pairing during meiosis. Both XX and XY cells have 2 PARs, implying equivalent effects of XX and XY PARs. Paradoxically, the process of X inactivation appears to spill over into the PAR and reduce expression on one X chromosome only in XX cells, leading to greater expression of PAR genes in XY cells compared to XX cells in the human transcriptome (30). A third potential source of X-linked imbalance stems from parentally imprinted genes in XX cells, which have one X chromosome from each parent and thus are influenced by any imprint on X genes from either parent. XY cells only receive imprints from the mother, and thus differ phenotypically from XX cells (37).

3. *XX mosaicism.* Female mammals are a mosaic of cells of 2 types: those expressing the X chromosome from the father (Xp), or from the mother (Xm) because of X inactivation (25). In contrast, XY individuals will lack this diversity within cell types in each organ because only one X (Xm) chromosome and only the maternal imprint of X genes will be expressed in each cell. The mosaicism in females means that in genetically diverse populations, the effects of disease-promoting X-linked alleles, inherited from one parent, will be muted in XX cells because half of the cells will have a different allele (38), and genomic imprints from each parent will only be expressed in half of the cells. In general, XX tissues are thought to have less extreme phenotypes than XY tissues, because the effects of extremely deleterious or beneficial alleles or imprints are buffered by the diversity of X alleles and imprints. For example, hemophilia A and hemophilia B (clotting factor VIII and IX deficiencies, respectively), are X-linked diseases that affect men, whereas most women are asymptomatic carriers.

Sexual Differentiation Caused by Gonadal and Nongonadal Hormones

In mammals, the process of reproductive system development requires the action of hormones (peptide/gonadotropins and steroids) from the pituitary gland, the adrenal cortex, and the gonads. Testicular development leads to secretion of Müllerian inhibitory hormone (MIH, also termed anti-Müllerian hormone, AMH), a glycopeptide, and testosterone, which affects many sex differences in nongonadal tissues (39). In contrast to the fetal testis, the fetal ovary makes minimal steroid hormones

(40), and ovarian function is not needed for development of the female reproductive system, as evidenced by the normal female anatomy of individuals with Turner syndrome, who have 45,X gonadal dysgenesis. The pioneering work of Alfred Jost suggested that 2 classes of testicular hormones are involved in sexual differentiation. First, testicular androgens drive the differentiation of the fetal external genitalia from female morphology to that of the male and are required for the differentiation of embryonic Wolffian ducts into male internal reproductive structures (41, 42). Androgens, secreted by Leydig cells, are required for the differentiation of embryonic Wolffian ducts into male internal reproductive structures (epididymis, vas deferens, ejaculatory ducts, prostate, and seminal vesicles), and drive the differentiation of the undifferentiated external genitalia toward male morphology. Second, the testis produces locally acting MIH that causes involution of the Müllerian ducts, which would otherwise develop into the fallopian tubes, uterus, and cervix (43, 44).

It was long thought that only the involution of the Müllerian ducts was an active process, with the Wolffian ducts simply involuting in the absence of androgens. Recent evidence from mice indicates that Wolffian involution is also an active process controlled by the transcription factor COUP-TFII (22, 45), but the nature of any factors stimulating COUP-TFII remains unknown (22). Some aspects of gonadal differentiation are active throughout life,

preventing ovarian follicle cells from transdifferentiating into “testis-like” cells (11). MIH is secreted by Sertoli cells and androgenic steroid hormones, usually testosterone, are secreted by Leydig cells. Testosterone and its more potent derivative dihydrotestosterone are responsible for the development of the male external genitalia (46). Androgens from adrenal glands and alternative pathway androgen biosynthesis in the human placenta can influence virilization of the developing fetus (47, 48). The adrenals of adult primates also produce abundant androgens, profoundly influencing phenotypes, so that not all sex steroids are gonadal (see Boxes 1 and 2). Although the term *sexual differentiation* is usually applied to the development of sex differences in genitalia and other organs such as the brain in the growing fetus; sex differences also occur later in life during the mini-puberty of infancy (49), puberty, the female menstrual cycle, menopause in women, and andropause in men. The actions of gonadal and nongonadal hormones as well as sex and autosomal chromosome gene products in adult people causes many sex differences in health and disease.

Influence of Gonadal Steroid Hormones and Nongonadal Hormones in Brain Development

Differentiation of the brain by gonadal hormones is implemented during a restricted critical window, which is operationally defined by the onset of copious androgen

Box 1. Steroidogenesis in gonadal and nongonadal tissues

All biologically active sex steroids, whether gonadal or nongonadal in origin, are derived from cholesterol by the process of steroidogenesis. Two steroidogenic steps must be considered (for details see (50)). First, the cholesterol side-chain cleavage enzyme, P450scc (CYP11A1) initiates steroidogenesis by converting cholesterol to pregnenolone; expression of P450scc renders a tissue “steroidogenic,” that is, able to make steroids de novo (51). The gonads, adrenals, and placenta express abundant P450scc and produce the familiar circulating endocrine steroids, but the brain, skin, and some other organs also express low levels of P450scc and produce steroids involved in paracrine actions. Brain steroidogenesis has been studied mainly in fetal rodents, with little information in other systems (52). Many nonsteroidogenic tissues (liver, kidney, fat, breast, heart) do not express P450scc but express other steroidogenic enzymes that modify steroids taken up from the circulation. Fat and breast express CYP19A1 (aromatase), permitting local production of estradiol from circulating 19-carbon (C19) steroids; this estradiol is important in breast cancer but is not a gonadal steroid. Similarly, prostate and genital skin express several enzymes leading to dihydrotestosterone, accounting for the failure of “androgen deprivation therapy” by gonadectomy in prostate cancer. Not all gonadal steroids are sex steroids, as both the ovary and testis secrete some “upstream” steroids that are precursors of the classic sex steroids. For example, dehydroepiandrosterone (DHEA) does not bind to sex steroid receptors, but it can be converted into testosterone and estrone. Second, synthesis of all sex steroids requires P450c17 (CYP17A1), which catalyzes 17 α -hydroxylation and the 17,20 lyase activity that changes 21-carbon steroids to C19 precursors of androgens and estrogens. P450c17 is abundantly expressed in the gonads of all vertebrates and in the adrenals of most vertebrates other than rodents, but the rodent *Cyp17A1* gene is silenced by tissue-specific methylation (53). Consequently, rodents make only miniscule amounts of adrenal C19 steroids and also use corticosterone instead of cortisol as their glucocorticoid. In most mammals, P450c17 has low 17,20 lyase activity, so that their adrenals produce rather small amounts of C19 steroids, but primate P450c17 has abundant 17,20 lyase activity, generating abundant C19 androgen precursors (DHEA, DHEA-sulfate, androstenedione) (47, 48). Furthermore, production of these C19 steroids proceeds by different pathways in rodents and primates: primates favor the “ Δ 5 pathway,” through DHEA, whereas rodents favor the “ Δ 4 pathway” through 17OH-progesterone (17OHP) (50). Primate adrenals also produce a true androgen, 11-keto-testosterone (54), profoundly influencing phenotypes (apocrine odor; female sexual hair). Thus, not all sex steroids are gonadal: ~ 50% of the circulating androgens in adult women are of adrenal origin.

Box 2. Gonadectomy and sex steroids

Many animal studies employ gonadectomy to eliminate the actions of sex steroids (estrogens, androgens, progestins). If using this approach, the investigator must consider whether nongonadal tissues will produce sufficient sex steroids to influence the study. The gonads produce most but not all circulating sex steroids; furthermore, some tissues produce steroids that act locally and do not enter the circulation, hence absence of a measurable steroids in blood does not ensure absence of its action in the target tissue. Both sexes produce all steroids and their metabolites, hence there are no male- or female-specific sex hormones, *per se*. In male mammals, testosterone release is highly pulsatile in nature (49, 55) and in laboratory mice, strain-dependent variations in androgen levels are reported (56). In female rodents, circulating levels of estradiol, testosterone, and DHT are highest in proestrus phase; a comprehensive analyses of sex steroids in intact and gonadectomized rodents can be found elsewhere (57). Circulating concentrations of testosterone in adult women are similar to those of boys in early puberty, and estradiol concentrations in men are similar to those in mid-cycle women, but the tenfold higher concentrations of testosterone obscure its effects. Rodents are widely used in research, but they differ from primates in several important aspects of steroidogenesis (see Box 1), and hence must be used with caution in studies seeking to model aspects of human physiology that might be influenced by steroids. These differences include: (i) In humans, substantial amounts of circulating sex steroids are bound to sex hormone-binding globulin (SHBG), whereas this carrier protein is not present in rodent circulation (58). (ii) Dehydroepiandrosterone (DHEA) and androstenedione, 19-carbon (C19) precursors for testosterone and estrone, that do not bind to sex steroid receptors, are secreted from the adrenal glands, the ovary and testis in humans, but not rodents (59). Thus, not all gonadal steroids are sex steroids. (iii) The rodent ovarian corpus luteum produces progesterone throughout pregnancy but in human pregnancy the corpus luteum involutes early in the second trimester, after which the placenta produces the progesterone needed to suppress uterine contractility, permitting term pregnancy. (iv) Adrenal-specific methylation of rodent *Cyp17A1* prohibits their adrenal synthesis of C19 precursors of sex steroids; however, changes in methylation status can occur under conditions of pathology. (v) As a further consequence of adrenal *Cyp17A1* methylation, rodents utilize corticosterone as their glucocorticoid, whereas almost all other vertebrates use cortisol. (vi) Rodent adrenals use high-density lipoproteins (HDL) taken up via scavenger receptor B1 (SRB1), as their principal source of cholesterol for steroidogenesis, whereas primates use low-density lipoproteins (LDL) taken up by receptor-mediated endocytosis. (vii) Several genes encoding steroidogenic enzymes are duplicated; rodents and primates differ in which copy(ies) of these genes are expressed: *CYP21*; *HSD3B*, *HSD17B*, *AKR1-3*. Such differences may affect laboratory results in unanticipated fashions. (viii) In rodents, nonsteroidogenic tissues such as the gut, liver, kidney, fat, breast, heart, thymus, skin, and the placenta have all been shown to make steroids. Thus, gonadectomy may eliminate most, but not all, circulating sex steroids, depending on the species being studied and may not reveal much about the paracrine effects of sex steroids present in the tissue(s) under investigation. Nonetheless, gonadectomy is an invaluable research tool that helps unequivocally confirm the influence of gonadal hormones in sex differences.

production from the fetal testis. Human fetal androgen production begins at 8 to 10 weeks postconception and in rodents is closer to parturition, at embryonic days 16 to 18, with birth following 2 to 4 days later. An important effect of this androgen surge is to masculinize the rodent brain. Steady but pulsatile release of the gonadotropins luteinizing hormone and follicle stimulating hormone from the pituitary gland support continuous steroidogenesis and production of sperm (60). In female rodents, the feminization of the brain proceeds in the absence of exposure to high levels of androgens or their aromatized byproducts, estrogens, a developmental strategy highly analogous to that used for masculinization of the gonads, reproductive tract, and secondary sexual characteristics, with the exception that estrogens are actively downregulated in male rodents. In human females, gonadotropins from the pituitary gland regulate ova development, induction of ovulation, and stimulation of estradiol and progesterone from the ovaries (49). An important feature of this developmental strategy is the existence of a sensitive period in female rodents (61). Male rodents must be exposed to high levels of

androgens during the critical period; if exposure occurs too early or too late it will be ineffective at inducing masculinization. However, females are also sensitive to androgens during a restricted period of development, hence a sensitive period in rodents. In males, the critical period closes shortly after androgen exposure because the cellular and molecular processes of masculinization have been initiated and cannot be reversed; the train has left the station. In both primates and rodents this process is largely prenatal, but female rodents remain sensitive to androgen exposure into the first postnatal week. Injecting a newborn female rodent with androgens will initiate the process of masculinization, thus she is still sensitive. After the first week, the feminization process cannot be overridden by androgens and thus the sensitive period has closed. The existence of the sensitive period in females is useful as a research tool—it is important in understanding the potential impact of exposure to endocrine-disrupting compounds or other cellular agents of masculinization that act in an analogous manner to androgen exposure in modulating female brain development. There is evidence for a later sensitive

period for brain feminization mediated by small increases in estrogens (62); this topic warrants further investigation. The closing of the sensitive period in primates, especially humans, remains poorly understood, but it appears to end prenatally, similar to the critical period in rodents. The sources of androgens that females can be exposed to during the sensitive period include from: (i) experimental interventions; (ii) male littermates in animals; (iii) or human adrenals carrying genetic mutations in the steroidogenic pathway (as in congenital adrenal hyperplasia).

Given that the critical and sensitive periods for sexual differentiation are defined by the production and response to gonadal steroids, it is not surprising that steroids are the primary drivers of developmental origins of sex differences in brain (and probably other tissues) and behavior. But how do steroids achieve this? The first step in any investigation is often to identify the active steroid metabolite(s). In rodents, circulating fetal testicular testosterone enters the fetal brain where it can serve as a direct precursor for estradiol synthesis via aromatase (*Cyp19A1*) (see Box 1). Fetal and adult neurons can aromatize testosterone to estradiol in a nonrandom distribution: neurons of the hypothalamus, preoptic area, and amygdala are particularly active for local estradiol synthesis, whereas the hippocampus and parts of the cortex, midbrain, and spinal cord are also active at a lower level (63). For most reproductive endpoints, it is the local actions of estradiol that drive neural phenotype toward masculinization, which to some seems counterintuitive, given that estradiol is so often referred to as a “female” hormone (64), and further highlights that it is impossible to completely eliminate the effects of sex steroids, especially in the brain, by simple gonadectomy (see Box 2). Developing rodent embryos sequester maternal estrogens by binding to circulating alpha-fetoprotein, which is present only during the critical/sensitive period; when it is genetically deleted, all the offspring are masculinized (65). However, in humans, sex hormone-binding protein, not alpha-fetoprotein, is the major serum glycoprotein that binds androgens and estrogens with an undetermined role in fetal sexual development (66, 67).

In rodents, there is abundant evidence that gonadal androgens are metabolized to estrogens in the brain and mediate “masculinizing” effects on the brain; similar evidence in primates is limited. In primates, the principal masculinizing agents are androgens, not estrogens, and although there is alpha-fetoprotein present in fetal circulation, it has a weak binding affinity for estradiol (68), and instead it plays a much broader role in brain and body development (69). The conclusion of no strong role for estrogens in humans is based on individuals with dysfunctional aromatase or androgen receptors. Males lacking aromatase still identify as men,

while XY individuals with complete androgen insensitivity identify as women (70). The disparity between the principal differentiating hormones in primates versus rodents suggests that findings may not be easily extrapolated, and it is important to specify both the hormone and species under investigation. To discern whether the biological basis of sexual differentiation of brain and behavior differs between primates and rodents, one needs to identify mechanisms by which steroids transduce signals to modify the trajectory of the nervous system. While those mechanisms are incompletely understood, a few general principles are clear. First, there is no unified mechanism that applies broadly across the brain, with the exception that androgens and estrogens are the primary drivers of masculinization during a restricted developmental window. Similar masculinizing effects of testicular androgens may also occur during puberty (71). Second, all aspects of neural development are capable of being “organized” or programmed by sex steroids. This includes cell genesis, migration, myelination, dendritic and axonal growth and branching, synapse formation, synapse elimination, and neurochemical differentiation. Effects are not limited to neurons, with both astrocytes and microglia also exhibiting morphological sex differences. Third, each discrete brain region, nucleus, or subnucleus appears to have unique mechanisms of cellular masculinization. In some brain regions, such as the preoptic area, there are multiple separate mechanisms at play simultaneously. Sex steroids act in both paracrine and endocrine manners to influence structural development and function (72, 73).

Biological Basis of Diversity in Sexual/Gender Development and Orientation

Given the complexities of the biology of sexual determination and differentiation, it is not surprising that there are dozens of examples of variations or errors in these pathways associated with genetic mutations that are now well known to endocrinologists and geneticists (74); in medicine, these situations are generally termed *disorders of sexual development* (DSD) or *differences in sexual development* (75). DSD includes genetic disorders in the sexual determination pathway (76), disorders of steroidogenesis (50, 77), disorders of steroid hormone action, especially androgen insensitivity syndrome (78), and less well-defined “developmental field defects” (79), such as Mayer-Rokitansky-Küster-Hauser syndrome (80). The study of genes and factors underlying DSD and the diagnosis and management of the various forms of DSD is a complex and rapidly evolving area of endocrinology: clinical management is complex (81) and requires both contemporary molecular genetics (82) and well-integrated interdisciplinary care (83).

Gender includes perception of the individual as male, female, or other, both by the individual and by society. *Gender identity* is a psychological concept that refers to an individual's self-perception; while associations between gender identity, neuroanatomic, genetic, and hormone levels exist, a clear causative biological underpinning of gender identity remains to be demonstrated. Both animals and human beings have biological sex, but only humans have evident self-awareness that allows them to express gender; self-awareness in animals has not been investigated in this context. Gender also includes differences that males and females experience in their social and physical environments, which can have differentiating effects on the sexes. Human social environments are poorly modeled in laboratory animals and thus animal studies are usually limited to addressing sex differences. For centuries, the concept of male and female did not distinguish between biological sex differences and those caused by consistent differences in the environments. Thus *sex differences* are those caused by biological factors, whereas *gender differences* reflect a complex interplay of psychological, environmental, cultural, and biological factors (Fig. 1).

At birth, individuals are assigned a sex or gender ("natal gender"), almost always based on the appearance of the

external genitalia. In most individuals, the various biological determinants of sex are consistent with one another, and this biological sex is also consistent with the individual's self-perception—the sex and gender are concordant. However, a substantial minority of people who do not have DSD have some degree of variation in their self-perception of their gender, which may differ from their biological sex; this is usually termed *gender incongruence* (84). The term *gender disorder* has been replaced with the term *gender dysphoria* which describes the distress that an individual might feel as a consequence of having gender incongruence. *Transgender* (often called *trans*) refers to individuals who do not identify themselves as being of their natal gender, whereas *cisgender* (*cis*) people do not experience gender incongruence (85). Readers are also referred to Endocrine Society's 2017 Clinical Practice Guideline and Transgender Health Fact Sheet (84). Estimates of the prevalence of male-to-female transgender individuals among general populations range from 0.5% to 1.3% and estimates for female-to-male transgender individuals range from 0.4% to 1.2% (85). State level population-based surveys indicate that 0.6% of US adults (25-64 years of age) and 0.7% of adolescents and young adults (13-24 years of age) identify as transgender. Other studies of US high school

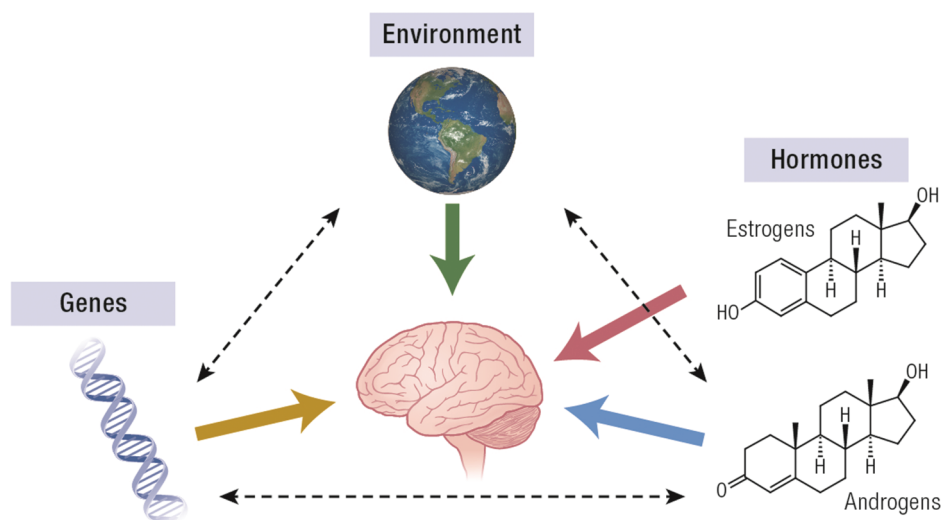


Figure 1. Simplified view of the factors influencing sex differences in the brain. Three broad groups of factors influence the sexually dimorphic brain, as indicated by the broad, colored arrows. 1) Genes and genetic factors that influence the brain include both those on sex chromosomes and autosomes, and include both the DNA itself (represented by the classic double helix) but also chemical modification of DNA (eg, methylation) and modifications of proteins associated with DNA to form chromatin, including histones, and also changes in proteins that bind to DNA. 2) Hormones clearly influence sexual dimorphism in the brain; these are represented by the principal sex steroids, estradiol and testosterone, but also include other steroid and protein hormones (progestins, MIH, oxytocin, prolactin, etc). 3) The environment includes a wide spectrum of influences, including perinatal nutrition and familial support, socioeconomic and demographic factors, intrinsic factors of brain development, age, and gender, and larger environmental factors, such as education, profession, and societal expectations (the "gendered environment"). In addition to each class of factor influencing the brain (bold arrows), the human brain also reciprocally influences each of these groups of factors. Furthermore, each group of factors influences the other, as represented by the dotted arrows. Some examples include: the environment influences genes via epigenomics and genes influence the environment by population sizes and domains; the environment influences hormones by seasonal variations and the actions of xenobiotics, and hormones influence the environment by promoting reproduction and consumption of foodstuffs; genes directly influence hormones by regulating their production and action, and many hormones, including all steroid hormones, regulate gene transcription.

students suggest a prevalence of 1.8% to 2.7% of being gender nonconforming or transgender (86-88). However, several factors may influence reported prevalence of gender dysphoria: (i) small sample sizes; (ii) differences in assessment techniques leading to incomplete ascertainment of gender dysphoric individuals; (iii) unwillingness of some individuals to respond fully and honestly, especially in older studies or studies deriving from locales where gender incongruence is a social taboo; (iv) differences in the subjects ages. *Sexual orientation*, not to be confused with gender identity, refers to the group of persons to whom an individual is sexually attracted; both cisgender and transgender individuals may be hetero-, homo-, or bi-sexual (89).

Although gender is strongly influenced by environmental and cultural forces, it is unknown if the choice to function in society in male, female, or other role(s) is also affected by biological factors (89-91). A general issue is that the association of sex, gender, or sexual orientation with specific brain structures, or with other biological variables, does not establish whether the biological variables are causes or consequences or noncausal correlates of the behavioral characteristics or function of the individuals studied. Three areas of biological difference have been studied fairly extensively: neuroanatomy, genetics, and hormones. Studies have reported differences in the hypothalamic INAH3 nucleus in men vs women and in homosexual vs heterosexual men (92, 93). Although initially controversial, others have confirmed sex differences in INAH3 numbers, not in size or densities, whereas no evidence for sexual dimorphism of any other INAH structures are reported (94). Studies in people with gender dysphoria found that the phenotypes of specific brain structures, such as the bed nucleus of the stria terminalis, of transgender women and transgender men differ from cisgender men and women, with partial, but incomplete sex reversal of sexually dimorphic structures (95). Brain networks involved in one's body perception, (pregenual anterior cingulate cortex, temporo-parietal junction, and fusiform body area) differ in individuals with gender dysphoria compared with cisgender individuals (96-98). Neuroimaging shows that testosterone treatment resulted in functional and structural changes in brain areas associated with self-referential and own body perception (99). Transgender men have thicker medial prefrontal cortex than cis men. Testosterone treatment does not change prefrontal cortex thickness in transgender men, but it has other effects on cortical thickness, connectivity, and fractional anisotropy (99).

Genetics may play a role in gender identity (100): monozygotic twins have 39% concordance for gender dysphoria (101). Attempts to identify specific genes governing gender identity have been plagued by small numbers of subjects and low statistical significance; no

specific gene has been reproducibly identified. However, such studies have suggested associations with genes encoding steroidogenic enzymes and sex steroid receptors, and it is generally agreed that androgens play an important but not determinative role. For example, many 46,XX individuals with severe virilizing congenital adrenal hyperplasia (steroid 21-hydroxylase deficiency) are exposed to intrauterine testosterone concentrations typical of those in normal male fetuses and consequently have severely virilized external genitalia; nevertheless, most have a female gender identity, but about 5% to 10% of such individuals have gender dysphoria, an atypical gender identity (89, 102, 103), or atypical sexual orientation and gender behavior (104, 105). Similarly, about half of 46,XY individuals with defects in androgen synthesis who were raised as females revert to a male gender role (106). The biological underpinnings of sexual orientation and gender identity are apparently related but are not the same (107). Thus, there is ample but incomplete evidence for biological substrates—neuroanatomic, genetic, and hormonal—for gender orientation, making this an important area of ongoing research.

Hormonal Versus Sex Chromosome Effects

Sex differences are caused by 3 major factors—sex hormones, genes on sex chromosomes/autosomes, and environment (Fig. 1). To understand disease mechanisms in both sexes and exploit sex differences in protection or exacerbation of diseases, it is important to determine the relative contribution of each of these factors in causing sex differences (17). Many sex differences caused by gonadal hormones have been discovered by measurements of sex steroids and gonadotropins during human development, and in animals by similar measurements or by interventional methods, such as gonadectomy, hormone administration, or the expression of synthetic enzymes or receptors in transgenic mice. Sex steroids play an integral part in many physiological processes (Box 1). Whereas the gonads are the major site of sex steroid synthesis, the adrenals, placenta, brain, and skin can also initiate steroidogenesis, and steroid-modifying enzymes are found elsewhere, especially in liver and fat, permitting synthesis of sex steroid hormones in multiple other sites (50). Thus, animal gonadectomy may provide information about endocrine effects of gonadal steroid hormones but cannot address tissue-specific paracrine effects (Box 2). Moreover, gonadectomy cannot mimic low pre-pubertal levels or physiological conditions in which hormone levels decrease, such as aging or menopause. Manipulations of human gonadal hormones are routinely used in contraception and in the management of sex steroid-dependent cancers (eg, breast, prostate). When

a sex difference is discovered in human disease, and modeled in animals, the investigation of possible hormonal causation of the sex difference is usually the first option considered.

To detect effects of sex chromosomes that cause sex differences, one can compare people who have differences in their sex chromosomes, revealing effects of X or Y chromosome number (108-110). These results strongly suggest direct sex chromosomal contributions to sex differences in cell function. Comparison of brains of XY patients with complete androgen insensitivity (who are phenotypically female), with brains of control XY males and XX females, suggests that cortical thickness and functional connectivity between the limbic regions and the cortex are influenced not only by testosterone actions, but by sex chromosome factors as well (111). However, changes in the sex chromosome ploidy also alter gonadal hormones, so it can be difficult to isolate sex chromosome effects not mediated by gonadal hormone effects. Circulating human embryonic/fetal sex steroid concentrations are poorly characterized, and the tissue concentrations are almost totally unknown. Another approach is to use mice to identify genes on the X or Y chromosome that act outside of the gonads to cause sex differences, and then seek evidence that the orthologous human genes cause human sex differences. Controlled experiments are possible in which XX or XY mice with comparable gonadal hormones can be compared. A frequently used model is the Four Core Genotypes (FCG) model, in which the testis-determining mouse *Sry* gene is deleted from the Y chromosome (creating the Y^- or “Y minus” chromosome) and inserted as a transgene on chromosome 3 (*Sry+*) (Fig. 2 and Box 3) (112). The utility and limitations of these models have been extensively discussed (113, 114).

Considering Sex and/or Gender as Variables in Health and Disease

Women and men differ in many physiological and psychological variables. It is important to establish the mechanisms causing such differences in health and disease, and to consider sex-related variables in studies of human health and disease. These variables include, but are not limited to, sex- and gender-related factors. The inability to control all variables in human studies means that it may be impossible to determine the relative roles of environment and biology in causing a difference between women and men, when both types of variable can influence the trait. Furthermore, while “gender expression/behavior” can be observed, “gender identity” can only be known by what an individual states. Thus, gender identity, *per se*, cannot be studied in animals. In human studies, it is unethical to selectively manipulate specific biological and environmental variables, and most currently available data derive

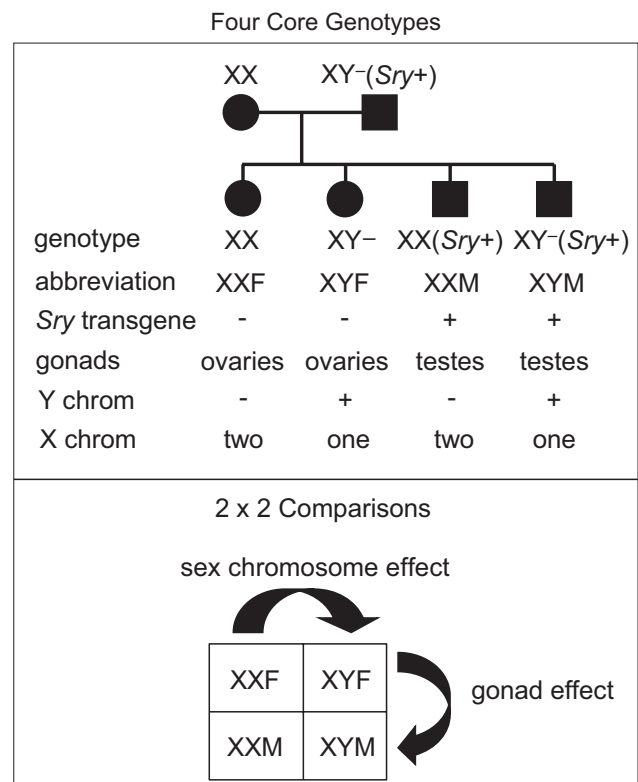


Figure 2. Schematic diagram of the Four Core Genotypes mouse model. The testis-determining gene *Sry* is deleted from the Y chromosome, producing the Y^- chromosome. An *Sry* transgene is inserted onto chromosome 3. Thus, the type of gonad is no longer linked to the sex chromosomes. The model produces XX and XY mice with *Sry* and testes, and XX and XY mice without *Sry*, with ovaries. Sex differences in phenotype can be attributed to an effect of gonadal hormones, comparing mice with ovaries and testes, or to an effect of sex chromosomes, comparing XX and XY mice with the same type of gonad. [Modified with permission from Arnold AP & Chen X. *Front Neuroendocrinol*, 2009; 30(1) © Elsevier Inc. (112)].

from studies comparing groups of men with groups of women. It is therefore difficult to disentangle the specific contribution of sex-related genes, hormones, gender-related variables, and other variables that contribute to being female or male. Because sex has long been defined by gonadal type, the list of sex-influencing factors has been primarily associated with gonadal hormones, especially estrogens, progestins, and androgens (121). However, some phenotypic sex differences develop before the gonads differentiate as testes or ovaries (122), so other factors also contribute to sex differences (123) but are seldom considered.

Sex is an essential part of vertebrate biology, but gender is a human phenomenon; sex often influences gender, but gender cannot influence sex. Studies of animal physiology must consider sex as a variable (124), with sex steroids (of both gonadal and nongonadal origins), sex chromosomes, and other factors contributing to sex differences in many physiologic processes. Similarly, studies of human physiology and disease must also consider sex for the same reason (125) and its disorders must

Box 3. Investigating sex chromosome complement versus gonadal hormones in health and disease: the four core genotypes (FCG) model

The FCG model allows for discriminating hormonal vs sex chromosome effects in animals. Gonadal males (XY⁻(*Sry*+)), bred to XX gonadal females, produce 4 types of offspring: XY⁻ and XX mice with the *Sry* transgene and testes, and XY⁻ and XX gonadal females lacking the *Sry* gene (Fig. 2). Thus, it is possible to compare XX and XY mice with the same type of gonad, in 2 separate comparisons. Differences between XX and XY are attributed to effects of sex chromosome genes acting on nongonadal tissues. To determine if this sex chromosome effect is caused by X or Y genes, a second model is studied, the XY* model (113, 114). This model produces genotypes that are similar to XO, XX, XY, and XXY. An effect of number of X chromosomes is discovered by comparing XO and XX, or XY and XXY. An effect of the Y chromosome genes is discovered by comparing XO and XY, or XX and XXY. These mouse models have been used to demonstrate sex chromosome effects causing sex differences in a wide variety of phenotypes and disease models, including brain and behavioral phenotypes, metabolism, autoimmune, cardiovascular and pulmonary diseases, Alzheimer disease, aging, and cancer (35, 113, 115). These models have facilitated discovery of several disease phenotypes in which the number of X chromosomes contributes to sex differences (116), and a smaller number of sex-biasing effects of Y genes (117). Sex chromosome effects occur in the same disease systems alongside sex-biasing effects of gonadal hormones, such that the 2 effects can synergize to increase the amount of sex difference, or counterbalance each other to reduce a sex difference. Moreover, genes encoded on the Y chromosome can have gene-specific effects, and/or effects that overlap with those of X genes (118). In the cardiovascular system and associated physiological/disease states, sex chromosomes and gonadal hormones can have opposing effects. Estrogens generally protect from cardiac ischemia/reperfusion injury and other cardiovascular diseases, reducing disease in female relative to male mice. However, studies of ischemia/reperfusion injury in gonadectomized FCG mice reveal that the XX sex chromosome complement is associated with worse outcomes, relative to XY (119). In another study, sex chromosome effects in angiotensin II-induced hypertension showed that arterial pressure was greater in gonadectomized XX mice than in gonadectomized XY mice (120). Sex chromosome complement also influences the development of abdominal aortic aneurysms, fat metabolism and adiposity, plasma lipids and lipoprotein levels (particularly HDL-C) (115).

also consider gender. However, human gender is a spectrum from feminine to gender-neutral to masculine, and also likely includes individuals who do not fit readily on a simple linear continuum (84). Studies addressing the endocrine care of transgender youth during the time of their potential gender transition (84, 89) find that they have a higher prevalence of stress-associated mental health disorders such as depression and anxiety, which can be ameliorated by gender-affirming endocrine treatment (126). It is essential to recognize these sex and gender differences as our health care systems endeavor to develop “individualized medicine.”

Despite the fact that biological sex is such a fundamental source of intraspecific variation in anatomy and physiology, much basic and clinical science has tended to focus studies on one sex (typically male). Few studies have done side-by-side testing for sex differences at baseline and in experimental models of human diseases (127-129). Studies in laboratory animals that manipulate biological (eg, genes and hormones) and environmental variables (eg, housing conditions, diet, physical activity, etc) demonstrate that many variables can affect sex-related aspects of an animal’s physiology. However, laboratory rodents may show male-female differences caused by different housing conditions, which could be misinterpreted as being caused directly by biological differences without environmental mediation. In studies concerning animal behavior, the sex and gender of the researcher conducting behavioral measures may also influence outcomes (130). Thus, for reproducibility and proper interpretation of the data, at the minimum, it is important to state the precise housing

conditions, anesthetics, analgesics (different effects in sexes), doses, surgical manipulations, diet, sex, strain, species, and age of animals used, as well as sex/gender of the researcher(s) performing experiments.

Having laid the foundation for several factors that contribute to sex versus gender, this Statement will use 3 areas of research as examples (not as a literature review) where human and animal sex differences are well known. First, sex differences in specific brain regions of healthy men and women are increasingly being documented along with differences in brain connectomes; these will be discussed in detail in Section II. Second, stress-related pathophysiologies are known to affect twice as many women as men. However, few studies systematically include study designs to ascertain function or mechanisms that may be similar or different between males and females. Hormones and signaling pathways that contribute to sex-specific differences in stress-based pathophysiologies will be discussed in Section III. Similarly, sex differences in manifestation of cardiovascular and renal diseases are well recognized and will be discussed in Section IV.

Section II

Developmental Origins of Sex Differences in Brain Anatomy, Function, and Behavior

Sex differences in the human brain are a topic of intense popular and scientific interest. Several scientific observations motivate the search for sex differences in brain structure

and function. First, the act of sexual reproduction requires that the male and female animals show qualitatively different reproductive behaviors. The stereotyped emergence of these reproductively critical and sexually differentiated behavior reflects biologically programmed (or “innate”) sex differences in the organization of those brain circuits that support the motivational and consummatory phases of copulatory behavior (131). Second, the fact that males and females make different biological investments in reproduction—eg, the risks of pregnancy in mammals are borne entirely by the female—sets up sex differences in the behavioral strategies that optimize reproductive fitness (132). Sexual selection based on sex-biased behavioral strategies is predicted to drive the evolution of sex differences in those brain circuits that are responsible for sexually selected behaviors. Third, males and females can show consistent sex biases in broader behavioral domains beyond those that directly relate to reproductive strategies. In our own species for example, there are highly consistent sex differences in the prevalence of physical aggression and violence (both male-biased) (133), as well as extensively documented sex differences in risk for different mental disorders (134).

In this section, we will first describe the main neuroimaging techniques commonly used in comparisons of brain anatomy, connectivity, function, and subnetwork organizations. We then review the key aspects of sex-biased brain anatomy and connectivity that have been revealed by these techniques; sex differences in stimulus-based or task-based functional magnetic resonance imaging (fMRI) studies are not addressed here. Next, we discuss specific disease states that appear to have different outcomes in the 2 sexes due to baseline differences in the “connectome” and animal models used in neuroimaging. Finally, we will address some important caveats and controversies in the field of brain imaging.

Brain Imaging Techniques

Modern neuroimaging methods make it possible to characterize diverse aspects of brain structure, function, and connectivity in vivo. This large toolbox of methods has been used to examine sex differences in brain organization at several levels of analysis. These techniques aim to analyze, map, and visualize regional and inter-regional (connectomic) features of the brain at macroscopic (systems-level) and mesoscopic (neural circuit architecture) levels in order to illuminate brain organization in health and disease (135). Of note, cellular-level details are beyond the resolution of most in vivo brain imaging techniques.

Sex differences in global and regional brain anatomy can be measured in vivo using structural magnetic resonance imaging (sMRI). Several considerations have made

sMRI an especially popular technique in the study of brain sex differences in humans. First, sMRI allows a quick and spatially comprehensive screen of the entire brain that can quantify thousands of morphometric properties simultaneously in vivo across a large number of individuals. These characteristics not only facilitate testing for sex differences outside defined regions of interest, but also allow longitudinal measurements that can track the emergence of brain sex differences over development (136, 137). Second, because sMRI considers structure rather than function, it can leverage evolutionary conservation of the basic mammalian brain plan (138), and it is therefore particularly well-suited for cross-species investigation of sex differences in humans and animals. Thus, a critical role for sMRI research in the study of brain sex differences is to screen for brain regions that can then be prioritized for closer analysis using more resource-intensive assays that are typically applied in a regionally selective manner.

Complementing sMRI, other in vivo neuroimaging techniques such as diffusion MRI (dMRI), resting state functional MRI (rs-fMRI), and fMRI provide unprecedented insights into tissue microstructure and brain connectivity. fMRI maps brain circuitry based on stimulus- or task-based brain functional responses. In contrast, rs-fMRI, by measuring changes in blood flow in the brain generated by signals dependent on blood-oxygen-levels, helps explore the brain’s functional organization by providing insights into intrinsic brain activity without requiring participants to be trained in specific tasks, thereby eliminating task performance as a confounder (139, 140). dMRI measures the differential patterns of water diffusivity in biological tissue revealing details of tissue microstructure, especially in white matter (141). Fiber tractography on dMRI enables mapping the fiber architecture of the brain, and subsequently, the network organization of the brain through structural connectomes (142-144). A brain connectome is an extensive map of the white matter structural or functional connections of the brain, created using dMRI or rs-fMRI (145). Modeling efforts, such as the Human Connectome Project, and the use of connectome-based predictive modeling, have provided an integrative, in-depth, and multilevel understanding of the structural and functional connectivity (regions that get coactivated) of the neuronal networks (146, 147).

Sex Differences in Global and Regional Brain Anatomy

It is well established that men have an average total brain volume that is approximately 10% greater than that of women (148, 149). A similar sex difference in average

human brain volume (~8%) appears to be present at birth (150) and is sustained throughout childhood and adolescence (151). The sex differences for total brain volume also hold for the 2 main subdivisions of brain tissue—gray matter and white matter—despite these 2 brain compartments following very different developmental trajectories (151, 152) (Fig. 3).

The robust sex difference in brain volume identified through human sMRI research cannot be fully explained by the fact that brain volume is positively correlated with height (average height is greater in men than in women). Statistical control for body size diminishes, but does not remove, sex differences in total brain volume (149), and boys also show greater average brain volume than girls during early adolescent development, at a time when girls are taller than boys (153). Thus, available literature supports a consistent picture in which there is overlap between the distribution of brain size in men and women, but the mean of this distribution is significantly greater in men than women. The medium effect size of sex on brain volume exists above and beyond sex differences in stature. However, it is important to note that no known functional sex differences associate with the sex difference in overall brain size. Sex differences in overall brain size, and their developmental timing, are both theoretically and methodologically important when considering: (i) whether neuroanatomical sex differences are conserved across species; (ii) whether there are sex differences in regional brain anatomy above and beyond sex differences in overall brain size; and (iii) whether

there is concordance between sex differences in brain size and any observed associations between brain size and putative biological causes of sex differences, such as gonadal or sex chromosome status (see below).

The patterning of sex differences in behavior and mental illness risk across the lifespan suggest that sex differences in human brain organization are likely to vary across different brain sub-systems or regions, and potentially also across different developmental periods. Structures in human gray matter compartments mediate neural computation and information processing—in contrast to axon-rich white matter compartments that are primarily involved in connectivity between different brain regions (see “Sex Differences in Brain Network Organization: The Brain Connectome,” below). Here, we focus on sMRI studies that have tested for sex differences in regional gray matter volume (regional GMV) after controlling for sex differences in overall brain size. Regional GMV sex differences that survive statistical correction for total brain volume variation are of special interest because they exist beyond global sex differences in brain size. We emphasize GMV rather than other morphometric properties of the brain such as cortical thickness, sulcation, or the shape of subcortical structures (144, 154), because GMV provides a common metric that can be examined across cortical and subcortical structures, with equal applicability to humans and mice. Independent large-scale human sMRI studies in biobanks have identified a reproducible pattern of sex differences in regional GMV using sample sizes that are

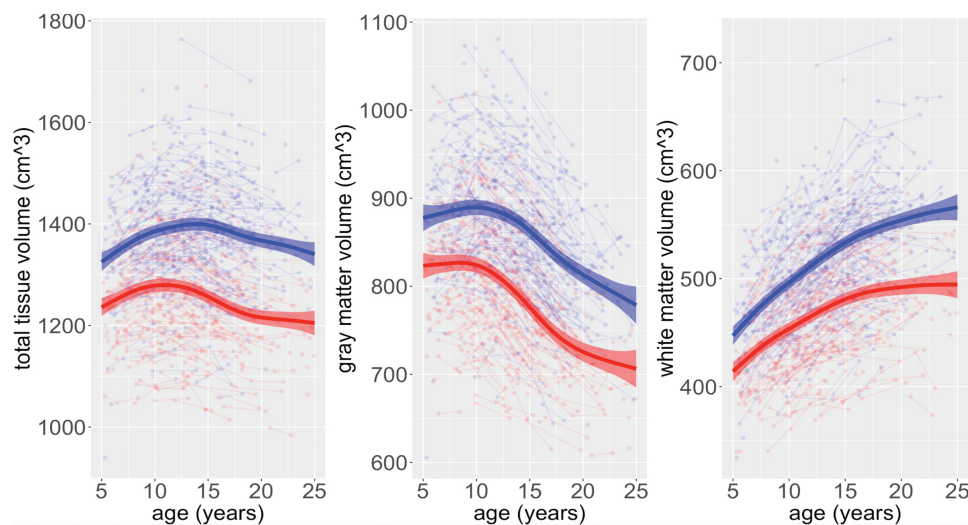


Figure 3. Developmental trajectories for total brain tissue volume, gray matter volume, and white matter volume in men and women over Development. Person-level data are shown for women (red) and men (blue) as points, with lines linking measures from the same person over time. Note the large interindividual variation in volumes within each sex, and the overlap of these distributions, between the sexes. Superimposed on these person-level data are group-level best fit volume trajectories (bold lines with shaded 95% confidence intervals). The developmental window covered is 5 to 25 years of age. For all plots, there are statistically significant sex differences in both trajectory shape (ie, sex differences in the tempo of volume change, $P < 0.00001$), and trajectory “height” (ie, sex differences in absolute volume across ages, $P < 0.00001$). [Adapted with permission from Giedd JN et al. *Neuropsychopharmacology*, 2015; 40 © Springer Nature (153)].

significantly larger than those used in earlier work (148, 149, 155). A structural neuroimaging study involving >2000 individuals demonstrated that higher regional expression of sex-linked genes was coupled with greater GMV in men relative to women (155). These studies, by different laboratories, using different datasets and different techniques for sMRI analysis, find a largely overlapping regional pattern of GMV sex differences after correction for sex differences in total brain volume. These independent replications of regional sex differences in GMV are also in agreement with meta-analytic studies (156). Together, these studies show that, in adulthood, regional GMV is (on average): (i) greater in women than men within superior parietal, dorsolateral frontal, and anterior cingulate cortices; and (ii) greater in men than women within occipital, fusiform, and parahippocampal cortices as well as the amygdala and putamen. Furthermore, while these studies lack temporally resolved developmental maps of male-female differences in regional GMV throughout the brain, there is extensive evidence from focused studies of particular structures that neuroanatomical sex differences can vary dynamically over development, such as observed with amygdala volume and shape (156).

The rapidly expanding body of sMRI research on regional GMV sex differences in the murine brain shows important overlaps and differences with findings from human studies (137, 157). These murine sMRI studies—which are most commonly conducted *ex vivo* at a spatial resolution of <100 μm throughout the whole brain—have been able to confirm the identification of all classically sexually dimorphic nuclei of male-biased volume from prior histological research, including the bed nucleus of the stria terminalis and medial amygdala (137, 157). These brain regions play a predominant role in modulating social and goal-directed behaviors, pain, and cardiovascular control, all of which are conserved among mammalian species and subject to sexually dimorphic outcomes. By allowing a full-brain screen, murine sMRI has also newly identified a reproducible set of regions with greater GMV in females, including the cerebellar cortex, ventral thalamus, and somatosensory cortex (137, 157). Furthermore, a longitudinal sMRI study in mice found that the set of regions with male-biased GMV can be detected by early postnatal life (with some accentuating over puberty), whereas regions of female-biased GMV in murine adulthood appear to emerge in adolescence (137). To date, there are no studies that formally seek to compare the spatiotemporal patterning of regional GMV sex differences in humans and mice, although existing work already suggests some potential homologies, including foci of greater cerebellar cortex GMV in females vs males by adulthood (137, 148) and the adolescent accentuation of male-biased amygdala volume (158, 159).

An important technical challenge in assessing the degree of anatomical homology between regions of sex-biased brain anatomy in humans and mice is that most of the best-established and histologically validated foci of sex-biased brain volume in mice (eg, bed nucleus stria terminalis, medial preoptic nucleus of the hypothalamus) are hard to image in humans due to their small size and intrinsic tissue contrast properties.

Sex Differences in Brain Network Organization: The Brain Connectome

The structural or functional brain network is represented by a “connectome,” wherein the structural or functional connectivity between coactivated regions is encoded either through fiber tracts or functional co-activations (160). These connectomes can be studied at the level of subnetworks like visuospatial, auditory, cognitive control, or macro-scale level through global measures of network segregation, integration, and efficiency, to obtain functional associations (161).

A study of 949 individuals (aged 8–22 years; 428 males and 521 females) showed that on average, there are significant differences between the sexes in their structural connectomes (Fig. 4) (162). On average, men had greater within-hemispheric connectivity, as well as enhanced network segregation, whereas between-hemispheric connectivity and network integration predominated in women (Fig. 4A), but these differences were most prominent during adolescence (Fig. 4B–4D). However, an opposite trend was seen for cerebellar connections, which developed differently between human males and females in adolescence and adulthood. The structural connectivity findings were consistent with a behavioral study conducted on the parent cohort (the above-mentioned imaging study was performed on a subset of participants), with women outperforming men on attention, word and face memory, and social cognition tasks, and men performing better on spatial processing and motor and sensorimotor speed tasks (163). An analysis of the Human Connectome Project rs-fMRI data identified age and sex as independent variables that contributed to differences in functional connectivity (164). In brains of men, functional connectivity was more clustered locally in all lobes, except in the cerebellum, whereas the brains of women showed a higher clustering coefficient at the whole-brain level. Thus, brains of men were classified as more segregated and brains of women as more integrated, which agrees with the structural connectivity findings (162). In connectomes, the identification of subnetwork properties (165) can reveal how the complex functional and behavioral repertoire emerges from the simultaneous processes of segregated neuronal clusters and their

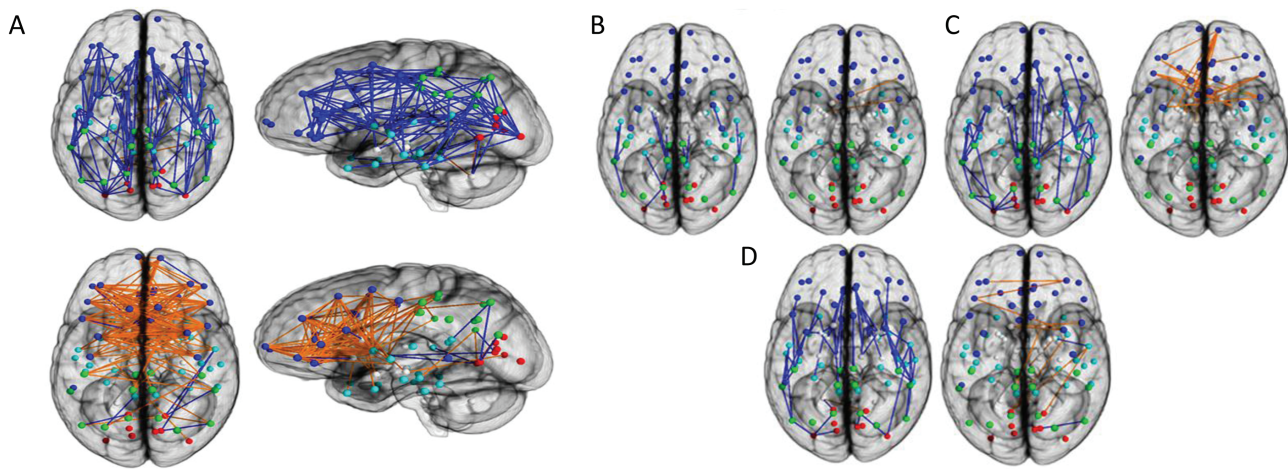


Figure 4. Sex differences in structural connectomes across development. Connectomes representing the white matter structural connectivity in the brain, with nodes indicating the brain regions and edges between the nodes representing the structural connectivity between the nodes. Node colors representing respective brain regions are as follows: dark blue, frontal; cyan, temporal; green, parietal; red, occipital; white, subcortical. The depicted edges shown are those that survived permutation testing at $P = 0.05$. **A**, shows increased intrahemispheric connectivity in men (Upper, in blue) and increased inter-hemispheric connectivity in women (Lower, in orange) on average. **B-D**: Connectivity differences shown in **A** separated by age groups are shown: **B**, under 13 years, **C**, adolescent (13-18 years), and **D**, young adults (18-22 years). Left image: Men/Boys; Right image: Women/Girls. [Adapted with permission from Ingahlhalikar M et al. *Proc Natl Acad Sci U S A*, 2014; 111(2) © National Academy of Sciences (163)].

integration during complicated cognitive tasks (166, 167). Consistent with the behavioral findings on sex differences, men had increased connectivity between motor and sensory (auditory) systems, along with increased connectivity in the fronto-parietal and cingulo-opercular systems that are traditionally associated with complex reasoning and control, whereas women had higher connectivity between reward, memory, and sensory (auditory) systems (163, 168). Better spatial skills in men and improved memory and social cognition skills in women have been reported in behavioral literature (169, 170).

It is important to point out that observed group-level differences in brain structure, function, or connectivity in men and women may reflect the influence of several extraneous factors. For example, in a set of elegant studies, brains of men were imaged to ascertain the contribution of performing complex spatial navigation tasks as part of their daily work on gray matter volume. These studies found that posterior hippocampi of London taxi drivers were significantly larger compared with controls (171), although the work did not address sex differences. Driving a taxi in London before the era of digital maps/navigation systems required extensive training and learning to navigate complex routes before being given a license to operate. In a subsequent study, comparison between London taxi drivers and bus drivers matched and controlled for age, education, intellectual, and stress levels, as well as years of driving experience, showed that taxi drivers had greater GMV in the posterior and less volume in the anterior hippocampi compared with bus drivers (172). Interestingly, years of

navigation experience associated with hippocampal volume in taxi drivers alone, but they were significantly worse at acquiring or retrieving novel visuo-spatial information than bus drivers. Importantly, no differences in other GMV, including the caudate nucleus, were found between the taxi and bus drivers; the caudate nucleus is associated with a myriad of cognitive and emotional functions. These studies illustrate brain plasticity and that professional work and years of performing certain tasks can result in brain structural, volume, and connectivity differences that may have little to do with sex or gender per se, but more with training, social environments, and behaviors. In other studies, GMV changes were greater in professional musicians, or after induced training (juggling for 3 months), and in early bilinguals, and white matter volume changes were found in adults learning a second language, irrespective of sex, when reported (173-176). These findings suggest that brain structure retains its plasticity and controlling for factors other than sex or gender are key in interpreting data on structural volumes and associated functions.

The above-mentioned existing datasets did not collect the requisite information on self-report of gender, thereby precluding retrospective analysis of gender in these cases. As identifying correspondence between behavioral scores and the regions that are involved in the manifestation of that behavior remains challenging, analyses of subnetworks pertaining to functional and behavioral domains can help elucidate a brain-behavior correspondence. The detailed description of sex differences in brain organization at the group level, and concerted efforts to specify

the role of sex-biased biological factors in shaping such sex differences, is of fundamental importance (177) and also provides a crucial adjunct for indispensable studies on environmental and wider societal contributions to sex-biased brain development. Such studies should be undertaken jointly using structural and functional connectivity. These studies elucidate the various ways in which sex differences in brain microstructure and connectivity can be investigated.

Sex Differences in Structural and Functional Brain Regions in Obesity

The hypothalamus has long been known as the “center” where peripheral and neural signals converge in the regulation of food intake and energy homeostasis in both sexes. Advances in neuroimaging studies have helped identify activation of several distinct brain regions comprising brain networks in response to eating in men and women. Behavioral and sociocultural factors may play a role in the observed sex differences in ingestive behaviors, appetite, and cravings related to obesity (178). Women report higher prevalence of maladaptive ingestive behaviors such as binge eating, food cravings, and “food addiction,” and the lifetime prevalence of disordered eating behaviors are about 3 times higher in women than in men (179, 180). Women also experience episodes of food cravings of greater intensity (181, 182), and greater frequency (183-185), and are less able to suppress food cravings than men (184, 186). Despite the wealth of data indicating that women experience disproportionately higher rates of food cravings, stress eating, and eating disorders than men, the reasons for these differences are incompletely understood (184, 187).

Regulation of food intake entails both homeostatic and nonhomeostatic factors (188). Homeostatic regulation balances energy needs with energy consumption, whereas nonhomeostatic regulation—in particular hedonic regulation and food addiction—involves reward-seeking behaviors that drive humans and animals to consume food beyond their metabolic needs, leading to the development of obesity (189-191). These findings have directed attention toward the extended reward system in obesity-related research, which consists mainly of basal ganglia regions and is involved in dopamine signaling and addiction-like behaviors (192). The extended reward system is composed of 6 interconnected brain networks—salience, central autonomic, basal ganglia, somatosensory, executive control, and emotional regulation (192).

Functional MRI studies have found that, in response to food images, obese individuals show greater activation than normal-weight individuals in regions associated with

reward anticipation, dopamine signaling, and addiction-like behaviors (193-196). Greater activity in brain regions of the extended reward network may drive obesity-related behaviors, such as greater responses to food odors and food consumption (197-199). Recent meta-analyses have further supported the role of the brain in disrupting the balance between energy consumption and expenditure. This combination of increased activity in regions associated with reward-driven behaviors and decreased activity in regions moderating top-down control of appetite may lead to consumption of excess calories (188).

Furthermore, sex-specific activations in response to food intake have been observed in cognitive, emotional, and reward-related regions (200-202). For example, obese men had greater activation than obese women in the supplementary motor area, precentral gyrus, fusiform gyrus, and inferior parietal lobule, which are associated with motor control, visuospatial attention, and responding to salient new or alerting stimuli (203). In this same study, obese women showed greater activation than obese men in the caudate and parahippocampal gyrus, regions implicated in reward processing and memory (203). Using graph theory to define the underlying architecture of brain structural connectivity obtained from diffusion tensor imaging, sex differences were observed in the topological measures of centrality (which determine the degree of information flow in specific brain regions) in regions of reward and salience networks in women, and in reward and sensorimotor networks in men (204). Resting state fMRI studies have found sex differences and commonalities in body mass index (BMI)-related connectivity associated with specific defined regions of interest in the reward network (205). For example, women had increased associations between BMI and increased connectivity in the in right globus pallidus and bilateral putamen. In men, BMI was associated with increased connectivity in the medial frontal cortex. A study of sex differences in response to visual and auditory food cues found that women experience greater activation in lateral and dorsolateral prefrontal and parietal cortical regions involved in cognitive planning and executive guidance and evaluation of behavior, compared with men (202). When viewed together, these studies highlight the importance of investigating sex differences in obesity-related alterations in the core and extended reward networks.

Although many single-sex studies of fMRI and obesity have been published, with the majority having all-female subjects, few studies have specifically investigated sex differences in brain function and structure in obesity. Despite the literature supporting sex differences in the brain, including in regions implicated in reward behaviors and energy homeostasis, few comprehensive reviews of sexually dimorphic brain signatures related to obesity have

been performed. A recent meta-analysis using an activation likelihood estimation approach to evaluate comparisons in functional responses to stimuli by obesity and by sex revealed differential sex- and BMI-related activations in reward anticipation and response, in shaping food-related memories, and in generating top-down control of appetitive processes. Together, these findings have important implications for sex-specific obesity treatments.

Models to Study Sex Differences in Normal Brain Structure and During Pathophysiology

Studies of sex differences offer important considerations for personalized medicine. The prevalence, clinical presentation, and symptomatic progression of many neurological and psychiatric disorders are remarkably different between the sexes. In addition to common X-lined mental retardation syndromes, men have a greater prevalence of neuropsychiatric disorders such as autism, attention-deficit/hyperactivity disorder (ADHD), and Tourette syndrome (206), whereas women have a greater prevalence of mood and eating disorders (207, 208). From the perspective of developmental disorders, the differences in the developmental trajectories of the sexes perhaps represent different vulnerabilities of maturing brain circuitry, leading to differences in symptoms, onset, and severity of neurological disorders. There are also sex differences in the risk factors, average age of onset, and prevalence of late-life dementias, as well as cerebrovascular disease (209). Additionally, in traumatic brain injuries, where the network organization of the brain is affected by the injury, such as the corpus callosum region, sex differences in inter-hemispheric connectivity and brain subnetworks may influence the impact of injury, and hence subsequent recovery. Thus, sex differences in brain connections are crucial to identify, as they may elucidate mechanisms in disease risk and potential treatment and recovery (210).

Most models of sex-biased mammalian brain development are based on experimental data from rodents (now largely from mice, but previously also from guinea pigs and rats). One of the most systematic dissociations of gonadal and chromosomal contributions to sex-biased anatomical brain organization in mammals is provided by a recent sMRI study of adult mice from the FCG model (112, 211). By combining sMRI with behavioral assays, these studies determined the contribution of sex chromosomes and gonads to adult mouse brain structure and function (211). This study revealed: (i) an effect of sex chromosomes on regional GMV in the cerebellar cortex and olfactory bulb; and (ii) an effect of gonads on regional GMV in the parietotemporal cortex and the bed nucleus of the stria terminalis. Some of these effects overlapped

with regions of normal sex differences in murine GMV (eg, cerebellar cortex and bed nucleus of the stria terminalis), and some brain regions were anatomically sensitive to both effects (basal forebrain and periaqueductal gray matter). Sex-chromosome effects on regional gray matter anatomy have also been reported by complementary sets of sMRI studies in both mice and humans that compare groups of euploid individuals with groups carrying X-chromosome aneuploidy (157, 212). Finally, in both mice (137) and humans (155), the spatial patterning of sex differences in regional GMV in adulthood appears to be preferentially aligned with the spatial patterning of sex-chromosome gene expression—which points toward a potential role of sex-linked genes in the establishment of maintenance of regional GMV sex differences. These studies emphasize the need for integrative models that view biological contribution to sex-biased brain development as a developmental dance of coordinated influences from both gonads and sex chromosomes.

Caveats and Critiques Relating to Neuroimaging of Brain Sex Differences

While several sMRI studies apparently establish that there are highly reproducible male-female differences in regional gray matter volume after controlling for variation in total brain size in humans, this conclusion should be considered in the light of several important caveats and critiques to avoid misinterpretation. First, all sMRI phenotypes that show reproducible and statistically significant sex differences also show a considerable overlap between men and women. This overlap is illustrated by total brain volume: total brain volume averages 10% greater in men than women, but many women have a total brain volume above the 30th centile for male brain volume, and many men have a total brain volume below the 30th centile for female brain volume (149). Sex differences in brain structure and organization are present across the lifespan and vary based on age, so inferences should be drawn cautiously. Thus, while total brain size shows a robust mean difference between men and women, an individual's total brain volume is a weak predictor of biological sex. These 2 facts arise because biological sex is only one source of variation in brain size (149), and other factors/variables that influence total brain size are unknown and/or hard to model statistically (Fig. 1). By extension, because sources of anatomical variation can differ between brain regions—the same individual can have GMV values that appear to be “sex-typical” in one region, but “sex-atypical” in another (when typical and atypical are defined by an individual's percentile position relative to the distribution of population-level trait variation in each sex) (213). This interpretation offers one

potential explanation for the observation that an individual brain can show varying degrees of GMV “sex-typicality” in different brain regions (relative to the population distribution). Alternative explanations have been proposed, including regional variations in programs of sex-biased development such that one individual’s brain may be considered a “mosaic” of male and female parts regardless of their chromosomal and/or gonadal sex (213).

Second, although sex differences in regional GMV are highly reproducible in humans and mice, these meso-anatomical sex differences *cannot* be assumed to correlate with behavioral sex differences. The functional relevance of neuroanatomical sex differences is hard to establish experimentally in humans, but correlations between anatomical and behavioral sex differences could be modeled in humans using several feasible study designs. To date, however, very few studies have directly tested for such structure-function correlations in humans (161), and this is an important priority area for future research. Several other challenges will need to be addressed in future work for any given sex-biased sMRI phenotype, including which aspects of behavior to measure and how to consider properly all possible configurations of brain-behavior association in 2 groups (eg, varying intercepts and/or regression slopes across groups). Moreover, some sex-biased sMRI phenotypes, such as trajectories of anatomical change, can only be estimated from group-level data, which complicates comparisons with interindividual variation in behavior. More fundamentally, however, regional GMV sex differences may be useful for understanding the brain basis for sex-biased behavior without GMV variation itself being the behaviorally relevant marker. For example, sex differences in mean regional GMV may help to define brain circuits that subservise sex-biased behaviors through their molecular, cellular, or connectivity features rather than through their volume *per se*. It is also important to entertain the possibility that sex differences in the anatomical organization of a given brain system may actually serve to equilibrate function between the sexes despite each sex having a categorically different genetic starting point.

Third, in addition to the functional considerations above, full understanding of a given sex bias in regional brain anatomy requires a mechanistic account that can link observed anatomical sex differences back to specific genetic and/or environmental factors that differ between men and women. It is usually impossible to disentangle biological sex differences from those which could be the result of environmental influences during development, differences in gender, and in sexual orientation

(Fig. 1). Strict causal tests for mechanistic models of sex-biased brain development are very hard to achieve in humans, although several informative approaches have been pursued including: (i) modeling sMRI data using normative variation in hypothalamic-pituitary-gonadal axis maturation or function (214); (ii) applying sMRI methods to cohorts undergoing gender-reassignment (215); and (iii) studying how sMRI features differ between typically developing groups and those affected by medical disorders involving the sex chromosomes (eg, sex chromosome aneuploidies) or sex steroids (eg, androgen insensitivity, congenital adrenal hyperplasia) (215, 216). However, the opportunistic and correlational nature of these approaches places considerable limits on the inferential power of mechanistic studies of human sex-biased brain development. Moreover, as challenging as it is to study chromosomal or gonadal factors in humans, it is even harder to address empirically the many plausible hypotheses about the potential for experiential and societal influences to differentially shape brain development in both sexes (121) or genders.

Section III

Sex Differences in Molecular Mechanisms Underlying Brain-Gut Disorders

The brain and the gut communicate with each other in a bidirectional way through parallel and interacting channels, involving immune, endocrine, and neural signaling mechanisms (217). The brain is able to modulate gut permeability, motility, intestinal transit, and microbial function via the autonomic nervous system (217), and the gut in turn sends signals to the brain to modulate behavior, in rodents (218). This brain-gut communication is especially critical in mediating stress responses and in stress-based disorders. In psychiatric and other neurological diseases, there are notable sex differences that point to different underlying neurobiological mechanisms in men vs women (219-221). Despite their clear documentation, these sex differences have largely been ignored, in order to develop broadly applicable pharmacotherapies that come at a considerable cost, especially for women’s health (222, 223). Sex biases in psychiatric risk are particularly instructive as they are developmentally patterned in a manner that is highly reproducible across different cultural settings and historical epochs: early-onset neurodevelopmental and gut disorders are more prevalent in boys than girls, while the opposite sex-bias is seen for adolescent-emergent mood disorders (134, 224). Brain-gut disorders are more prevalent in women than men, but this may be due to underreporting by men due to social stigma associated with several of these

disorders. The etiologies and risk factors for several brain-gut disorders differ between the sexes, yet study designs include predominantly male sex. In this section, we discuss the possibilities that shared and distinct mechanisms operate in males and females resulting in similar as well as distinct manifestation of symptoms for a given disease/disorder.

Sex-Related Differences in Obesity

Although prevalence rates for obesity are at unprecedented levels in all ages (225) and are almost equal in men and women (except when stratified by race or ethnicity) (226), recent surveys indicate an increase in the incidence of obesity in adults and sex differences in the associations between weight, physical health, and psychosocial functions (227, 228). Sex differences in body fat distribution have also been observed (178, 229), with women showing an increased propensity to gain total body fat, especially subcutaneous abdominal fat, whereas men tend to have more visceral adipose fat (230), which is associated with higher risks of type 2 diabetes, hypertension, dyslipidemia, and cardiovascular disease (231). Most clinical trials do not report sex differences related to health outcomes or treatment responses, but a few existing reports suggest women are less likely to complete treatment, tend to lose less weight than men, have a greater number of unsuccessful attempts to maintain weight loss resulting in the well-known “yoyo” diet phenomenon, and have limited responses to pharmacological treatments (225). Obesity-related studies in humans and rodents have expanded in scope to not only focus on structural and functional brain differences between obese and lean male and females, but also include investigations into the bidirectional signaling associated with the brain-gut microbiome axis (232, 233). In obese individuals, changes in the relative abundance and gut microbial diversity have been linked to changes in metabolism, insulin resistance, inflammation, and fat deposition (234). The importance of the intestinal microbiome to human health has been of interest over the past few decades, with multiple studies now linking the microbiome to energy homeostasis, immune function, and development of obesity and metabolic syndrome (235-237), even though few studies have addressed causality.

Not only does the brain-gut axis demonstrate changes in obese individuals, but evidence also highlights differences in the microbiota based on sex hormones (238). More recently, the effect of sex hormones on the composition of the gut microbiota has been explored, with differences seen in the microbiota between men and women during various stages of human development and maturation (238). These

sexually dimorphic microbiome signatures are likely to contribute to differences in susceptibility to autoimmune and metabolic diseases between the sexes. Studies performed in immunocompromised mouse models have shown delayed onset and lessened severity of type 1 diabetes in female mice who receive male microbiota transplants; testosterone activity and androgen receptor signaling was essential for this protection (239, 240).

These sex-specific differences in the microbial communities persist throughout adult development, with murine models demonstrating the role of testosterone in orchestrating these divergences in host selection of microbial communities (240). In rodents, males exhibit lower microbiome variability relative to females, likely due to the pulsatile nature of estrogens (240). Human studies comparing the microbiome of twins also revealed more divergences in microbial composition in opposite-sex versus same-sex twins (241). When the cecal contents from adult male mice is transferred into female mice, metabolomic profile changes and masculinization of the hormonal profile results, suggesting the gut microbiota's influence on sex-specific metabolic and behavioral phenotypes (239, 242).

Circulating estrogens in the body are metabolized by the liver and undergo methylation, hydroxylation, and conjugation reactions to produce metabolites that affect host metabolism (243). Certain metabolites are excreted through the bile and are further processed by microbial enzymes in the distal small and large intestine. Certain microbial species secrete beta-glucuronidase, an enzyme that deconjugates biliary estrogen metabolites and allows for its reabsorption into the bloodstream to act on distal sites through binding of estrogen receptors (244). Dysbiosis and decreased microbial diversity result in decreased production of absorbable estrogen metabolites. This mechanism has been implicated in pathologies associated with low circulating estrogens, such as obesity, metabolic syndrome, cardiovascular disease, and cognitive decline in women (245, 246); however, estrogen replacement therapy does not reverse these conditions (247). Growth hormone similarly contributes to sexually dimorphic responses in the above-mentioned diseases (248). In addition, estrogens modulate inflammatory pathways driving disease processes such as nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes (249, 250). More specifically, estrogens regulate adipokines and lipopolysaccharides, which respectively are adipocyte-derived hormones and endotoxins that have been associated with type 2 diabetes (251). Adipokines play a role in metabolic homeostasis as well as in mediating the beneficial and detrimental effects of inflammation (252). The androgen- and estrogen-dependent regulation of adipokines, including leptin, resistin, adiponectin, and visfatin, provides a possible mechanistic link between metabolic disorders (obesity,

atherosclerosis, insulin resistance) and autoimmune dysfunction. The estrogen-microbiome axis can provide a potential avenue for a sex-specific approach to combating metabolic disorders and highlights the bidirectional interaction of estrogens and microbial communities in the pathogenesis of disease processes.

Although the exact signaling mechanisms underlying the communication within the brain-gut-microbiome axis remain incompletely understood, tryptophan metabolites have been implicated as important signaling molecules (253). The most extensively studied tryptophan metabolite is serotonin (5-HT), a molecule with diverse roles in both the gastrointestinal tract (ie, peristalsis, secretion, and absorption) and the central nervous system (ie, mood, pain modulation, behavior, sleep, and ingestive and cognitive functions) (254). Tryptophan also acts as a precursor to the kynurenine (KYN) family of molecules (255). In obesity, the KYN pathway is preferentially activated and may contribute to immune-mediated inflammation, which may drive inflammation-associated changes to the extended reward network described in previous brain studies, particularly changes involving the amygdala and lateral orbitofrontal cortex (256-259). KYN may also modulate signaling within the brain-gut-microbiome axis through downstream neuroactive metabolites, such as kynurenic acid and quinolinic acid, functioning as N-methyl-D-aspartate (NMDA) antagonists and NMDA excitotoxins, respectively (260). Sex differences have been reported in these metabolite products in obese individuals, with lower tryptophan levels but elevated KYN and KYN/tryptophan ratios in women with high BMI compared to men with high BMI (256, 261, 262).

Sex Differences in Stress-Based (Patho) Physiologies

Epidemiological data reveal that the majority of psychiatric disorders occur at different rates in men and women. For example, men are more likely to suffer from attention-deficit/hyperactivity disorder (ADHD), whereas women are more likely to suffer from major depression and posttraumatic stress disorder (PTSD) (219, 263-265). Even when the rates of disorders are similar, their presentations can differ. Schizophrenia, for example, is only slightly more common in men than women, but men develop schizophrenia at an earlier age and present with more negative symptoms, such as social withdrawal and lack of motivation. (224). In the case of bipolar disorder, rates are similar between the sexes, but women more often have more rapid cycling and mixed episodes and they report higher comorbidity with eating disorders and PTSD, whereas men report higher comorbidity with alcoholism (266). Not only does the risk

and presentation of psychiatric disorders vary between men and women, but there are differences in treatment responses. For example, the efficacy of antidepressants differs between the sexes: men respond better to tricyclic antidepressants, whereas women respond better to selective serotonin reuptake inhibitors (267, 268). These findings implicate neurobiological sex differences in contributing to disease. In support of this idea, recent studies using animal models are beginning to uncover molecular processes that can bias males and females toward different pathology. Findings from some of these basic research studies will be highlighted here as examples of how including sex as a biological variable can inform our understanding of the etiology of stress-based disorders, as well as guide the development of better treatments.

While there are sex differences in rodent studies in the structure and the size of certain brain regions that can contribute to sex differences in behavior (211), imaging studies that focused on sex differences in cortical thickness and gyration suggest a role for these brain regions in humans as well. In adolescent girls, cortical thinning in the right temporal regions, the left temporoparietal junction and the left orbitofrontal cortex is faster than in boys (154). In contrast, changes in cortical folding were only found in one cluster of the right prefrontal region, suggesting that the mechanisms underlying changes in cortical thickness and gyrification in adolescents are distinct. Sexual dimorphism in the developmental course of the cortical maturation, which coincides with the onset of puberty, might explain sex differences in the age of onset and clinical presentation of many psychiatric disorders (154). Recent evidence has revealed that molecular sex differences in the brain are more widespread than initially thought and such seemingly small-scale differences can have a large impact on physiology and behavior (269). Neurons typically communicate with each other via neurotransmitters and neuropeptides, which are released from a presynaptic neuron and travel across a synapse to bind to receptors on the postsynaptic neuron to exert downstream cellular effects. There are sex differences in production and release of many neurotransmitters and neuropeptides that can result in behavioral changes. In other instances, sex differences in these systems are compensatory, leading to similar behavior endpoints via different mechanisms. For example, both male and female juvenile rats play, but the release of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) into the lateral septum mediates juvenile play only in female rats (270). There are also sex differences in receptors that can influence how these neurochemicals affect their downstream targets. For instance, dopamine 1 (D1) receptors, which belong to the family of G protein-coupled receptors (GPCRs), in the nucleus accumbens, are necessary for social

withdrawal in female but not male California mice (271). The function of GPCRs is often complex and they can induce different downstream effects depending on their conformation and location. Sex differences can occur at each level of receptor function, in some cases altering physiology differently in male vs female rodents. Sex differences in GPCR signaling are particularly important to consider, especially given that GPCRs are the most studied drug target family for a myriad of indications; in fact, 34% of all US Food and Drug Administration (FDA)-approved drugs are targets of GPCRs (272). As an example of the myriad of sex differences that can be mediated by receptors, we will use the corticotropin-releasing factor 1 and 2 (CRF₁ and CRF₂, respectively) receptors that facilitate responses to stress, exhibit sexually dimorphic expression pattern, are modulated by both estrogens and androgens, and have been relatively well characterized in both sexes (273, 274).

Upon perception of stress or perturbation of homeostasis, CRF is synthesized in the paraventricular nucleus and released from the median eminence of the hypothalamus into the pituitary portal circulation, which in turn stimulates the synthesis and secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the general circulation. ACTH acts on the adrenal cortex to stimulate the synthesis and release of glucocorticoids and other steroids. This activation of the HPA axis in the classic “flight or fight” response by the CRF system is present in all mammals. The mammalian CRF family comprises 4 agonists, CRF and 3 urocortins (UCN1-3); and 2 known class B GPCRs, CRF₁ and CRF₂. While CRF₁ and CRF₂ share ~68% identity at the amino acid level (275), they perform distinct functions; CRF binding to CRF₁ initiates stress responses by activating the HPA axis, whereas UCN1-3 binding to CRF₂ brings systems back to homeostasis (274). Not surprisingly, perturbations in the components of the CRF family impact several organs and lead to brain-gut disorders, type 2 diabetes, metabolic syndrome, cardiovascular, and reproductive diseases, among others (274). There are sex differences in CRF’s endocrine effects. In female rats, higher levels of CRF mRNA in the paraventricular nucleus are reported that associate with the estrous cycle (276, 277) and are reviewed elsewhere (274). Perhaps as a compensatory response, CRF binding protein, an endogenous protein that sequesters CRF thus preventing its bioavailability, is expressed at higher levels in the pituitary of female compared with male mice (278). In humans, there is evidence for increased CRF receptor sensitivity at the level of the pituitary of women relative to men, because peripherally administered CRF, which acts at the pituitary, increases ACTH to a greater degree in women (279).

During stress, CRF is also released centrally into many brain regions, where its neuromodulatory effects coordinate cognitive and behavioral changes to promote stress coping (280). There are sex differences in the way these brain regions respond to CRF that are largely due to sex differences in CRF receptor signaling (274). For example, there is greater CRF₁ receptor binding in the basolateral amygdala in female rats (281). In contrast, binding of the CRF₂ receptor subtype, which is involved in stress recovery, is greater in the central nucleus of the amygdala in male rats (281). It is unknown precisely how these sex differences affect behavior, but given that the amygdala is critically involved in fear, it is likely that these receptor sex differences differently alter fear processing in males and females. In the brain, CRF₂ is most abundant in the bed nucleus of the stria terminalis, a region that regulates sexual behavior and stress-related functions (282, 283). Promoters in genes for CRF₁ and CRF₂ receptors harbor estrogen and androgen responsive elements and show tissue-specific modulation by sex hormones (284, 285). The sexually dimorphic expression pattern of these receptors at normal physiological states and during stress or disease pathology are summarized in a recent review (274).

Sex differences in CRF₁ receptor signaling have been identified in the noradrenergic-containing nucleus of the locus coeruleus (LC) and these differences have important implications for understanding disease vulnerability (273). The LC-noradrenergic system regulates levels of arousal such that higher levels of norepinephrin are associated with greater levels of arousal (286-289). Stressor exposure causes CRF to be released into the LC, which speeds up LC neuronal firing, increasing norepinephrin release (290, 291). Activation of this system during an acute or moderate stressor is thought to be adaptive, because it is important to be alert during a stressful event. However, if this system is activated inappropriately or persistently it can lead to hyperarousal that contributes to agitation, restlessness, impaired concentration, and sleep disturbance. Hyperarousal is a key feature of PTSD and reported in a subset of depressed patients (292, 293). Similar sex differences in spatiotemporal expression of CRF₂ and its ligands are found in humans with gut disorders, where they could contribute to differences between males and females in vulnerability to brain-gut disorders (127, 294).

There are sex differences in CRF₁ receptor signaling in the LC that increase female sensitivity to CRF. In the LC, CRF receptors primarily couple to Gs to initiate signaling through the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling pathway (295-297). Sex differences in CRF₁-induced cAMP-PKA signaling are linked to greater coupling of the CRF₁ receptor to Gs in females compared to males (298). This sex difference in

coupling of Gs may indicate that the CRF₁ receptor has a different conformation or binding partner in females vs. males, permitting different proteins to preferentially bind in each sex. Further support for this idea comes from studies demonstrating that, in male rats, acute swim stress increases the binding of a different protein, β -arrestin2, to the CRF₁ receptor, and this effect is not observed in female rats (298). The increased β -arrestin2 in male rats likely contributes to the greater CRF₁ receptor internalization in stressed males (298). When taken together, these findings suggest that CRF₁ receptors preferentially signal through different pathways in males (small GTPases) and females (cAMP-PKA) (299). This difference in signaling could alter physiology and disease risk. In fact, sex differences in CRF₁ receptor signaling in cortex were linked to increased Alzheimer-related pathology, including increased tau phosphorylation and amyloid β signaling in female compared with male mice (300). Few studies investigate sex differences in GPCR signaling, but it is likely that sex differences in GPCRs are also found in receptors other than CRF and that these differences could confer vulnerability and resilience to many diseases.

In human studies, single nucleotide polymorphisms in the CRF receptor gene (*CRHR2*) are associated with negative emotions in patients with irritable bowel syndrome (IBS) (301). Immune cells secrete CRF₂ in extracellular vesicles that circulate in the plasma and associate negatively with disease severity scores in IBS-diarrhea patients (294). Single nucleotide polymorphisms in *CRHR2* are also associated with lifetime PTSD in women (302) and with type 2 diabetes (303). The prevalence of type 2 diabetes and insulin resistance is greater in men (304). Epidemiological studies have shown that men with high levels of self-reported perceived stress have a 1.4 higher odds ratio of developing type 2 diabetes during a 10-year follow-up period and are at 2-fold higher risk of developing diabetes than women with similar levels of reported stress (305). In agreement with human data, male mice lacking functional stress receptors (*Crhr2*^{-/-}) and haploinsufficient (*Crhr2*^{+/-}) mice have worse glucose and insulin tolerance, microvesicular hepatic steatosis, and dyslipidemia than female *Crhr2*^{-/-} or C57BL/6 male and female mice in a high-fat diet-induced model of diabetes (129). Female *Crhr2*^{-/-} mice had significantly greater brown adipose fat mass on high-fat diet than C57BL/6 female or male mice of either genotype, suggesting greater thermogenic responses that might be protective. However, the mouse study did not address whether steroid hormones contributed to changes in adipose mass or function. Thermogenesis in brown adipose tissue in humans in response to a meal or cold stress suggests that women have greater thermogenic responses

than men and that these responses correlate positively with progesterone levels, but negatively with cortisol levels (306). Thus, analyzing data from both sexes provides insights into sex-specific mechanisms that regulate physiological processes in both sexes.

In colonic tissues of pediatric patients with Crohn's disease, subcellular localization of CRF₂ differs between boys and girls (127). Furthermore, lack of CRF₂ revealed several sex-specific signaling pathways and differential degree of inflammatory responses in male and female mice (127). Treatment with UCN1, a high-affinity agonist for both CRF receptors, rescued *Crhr2*^{-/-} male mice from colitis-induced mortality, whereas UCN1 treatment increased mortality in *Crhr2*^{-/-} female mice (127). Both diabetes and Crohn's disease show sex differences in disease prevalence and outcomes, yet most animal studies use male sex to delineate mechanisms. Analysis of the data by segregating the 2 sexes can reveal significant insights into distinct and shared mechanisms and factors that exist at baseline and during disease. For example, sex differences exist in the etiology of pancreatitis: alcohol and tobacco predominate in men, whereas idiopathic and obstructive etiologies predominate in women (307), yet to date only a few studies have used both sexes to study mechanisms involved in pancreatitis. While both males and females develop pancreatitis in animal models, when administered identical doses of the pancreatic stressor caerulein, C57BL/6 female mice show less severe pancreatitis and histological damage than male mice (128). Lack of CRF₂ rendered female mice more susceptible to caerulein-induced pancreatitis compared with male *Crhr2*^{-/-} mice (128), with both male and female *Crhr2*^{-/-} mice exhibiting similar levels of total histological damage (128). Detailed analysis of components contributing to histopathological damage showed that female C57BL/6J mice have less necrosis, zymogen granules, and vacuolization than male mice with pancreatitis, but they have similar levels of edema and neutrophil infiltration as male mice (128). This data segregation allowed isolation of factors that differentially contribute to histological damage, which otherwise would be lost, if grouped together in this analysis. Taken together, these data support a role for the CRF receptors, product of an autosomal gene and regulated by steroid hormones to bring about sex-specific cellular signaling and function.

Sex Differences in Pharmacotherapy of Stress-Based Diseases

Sex differences in GPCR signaling are also relevant for pharmacology. Biased ligands can shift signaling toward

β -arrestin pathways and away from G-protein-mediated pathways based on how they bind to the GPCR (308). These biased ligands are being designed with the hope of providing more targeted therapies with fewer side effects (308, 309). Understanding sex differences in signaling and how such differences contribute to changes in physiology can inform the development of these biased ligands. For example, a CRF₁ receptor ligand that biases signaling through β -arrestin pathways may be useful for treating hyperarousal symptoms or reducing the progression of Alzheimer disease, especially in women. An idea for such a compound would never have come about if women were excluded from preclinical and clinical studies on CRF₁ receptor function.

The idea of using CRF₁ antagonists to treat depression, PTSD, and irritable bowel syndrome has been around for decades, but these compounds were ineffective in several clinical trials (222, 310). Sex differences in CRF₁ and CRF₂ receptor signaling may also explain the failure of different selective CRF₁ antagonists as treatments for these disorders. While there are likely many reasons for their failure, critical ones could be sex differences in their target, association of CRF receptors with different binding partners in female versus male cells, or heteromerization of CRF receptors (311-313), all of which can result in altered signaling. The consistent efficacy of CRF₁ antagonists in reducing anxiety-like and depressive-like behavior in rodents and nonhuman primates was established in studies primarily conducted in male animals (222, 314-317). In a study in which females were included, local blockade of CRF₁ receptors in the dorsal raphe with an antagonist reduced anxiety in male but not female mice, highlighting sex differences in efficacy (318). Yet these compounds developed primarily in male rodents were tested in clinical trials with participants of both sexes or only in women. Notably the only CRF₁ antagonist study that had success in reducing depressive symptoms, NBI-34041, was conducted only in men (222, 319). The approach of developing compounds in male animal models is not unique to CRF₁ antagonists and has been common practice (222). Collectively, these studies suggest that a failure of certain therapeutics may result from ignoring sex differences in their targets. Sex differences in targets are not well known because most preclinical studies use only male rodents (320, 321). Excluding females in the drug development stage particularly impacts women's health. Indeed, it is likely that some compounds deemed ineffective in male rodents would work in females, yet such compounds never would have a chance to make it to market, because of testing exclusively in male subjects. Moreover, the fact that most

drugs are designed using males also likely contributes to the higher rates of adverse drug reactions in women compared to men (322).

Including both sexes in mechanistic studies is critical for developing drugs that work efficaciously in both sexes (see Box 4). Latent sex differences can also impact drug development: a compound targeting a mechanism in men may not work in women. As the field moves forward, we may find that sex-specific therapeutics based on understanding latent sex differences are required to truly improve patient outcomes. In sum, there are observable sex differences in behavior that extend beyond reproductive function. Molecular sex differences in several organs, such as the gut and the central nervous system, play a key role in driving these functional and behavioral differences. Moreover, even when function and behavior are consistent between the sexes, the underlying processes can differ. Thus, including both sexes in preclinical molecular studies guiding drug development is key for improving the health of men and women.

Section IV

Sex Differences in the Cardiovascular-Renal System

Cardiovascular disease (CVD) is the major cause of premature death in both sexes worldwide, although women generally develop CVD 10 years later than men (328). In 2016, ~18 million people died from CVD, representing ~30% of all deaths worldwide (329). There are marked sex differences in CVD and renal disease. For example, women are protected from heart disease during the reproductive years but are more likely to die in the first year following a cardiovascular event than males (330). Most heart conditions, including myocardial infarction, Takotsubo syndrome, and cardiac arrhythmia, exhibit sex differences in symptoms and severity (331). Chronic kidney disease (CKD) is more prevalent in women but, once established, progresses more rapidly in men (332). However, this female advantage is lost after menopause. These sex differences in cardiovascular and renal disease have long been overlooked and underappreciated. The clinical presentation, the response to pharmacotherapies, standard care practices, and the underlying pathophysiological mechanisms differ in women compared to men. Furthermore, lack of understanding of sex differences in mechanisms underpinning cardiovascular and renal disease has led to poorer outcomes in women than in men. A major problem is that mechanistic preclinical studies in animal models have largely been conducted in males (333). Yet, it has become increasingly clear that sex differences

Box 4. Sex differences in pharmacokinetics and pharmacodynamics of drugs

Thalidomide, a sedative that was prescribed to many pregnant women to relieve pregnancy-associated nausea, was first sold in Germany (without a prescription) in 1957; it had been tested in animals and in men, but not in women. It was soon noted to cause multiple birth defects, most notably phocomelia (arrested limb development) and postnatal deaths. Fortunately, it was never approved in the United States, but thousands of children were affected around the world. In 1962, the US Congress passed the Kefauver-Harris Drug Amendments Act requiring manufacturers to prove a drug is both safe and effective (323). Consequently, the US Food and Drug Administration (FDA) recommended against drug testing on women, particularly those of child-bearing age, until the early 1990s. To date, most treatment guidelines are based on results from clinical trials conducted on middle-aged men. Dosage, pharmacokinetics, and pharmacodynamics data for women (and children) are lacking for most drugs. Activities of cytochrome P450 (CYP) enzymes show significant sex differences in drug metabolism in Phase I clinical trials (324). Gastric enzymes involved in oxidative degradation such as alcohol and aldehyde dehydrogenases are significantly more active in men than in women resulting in higher bioavailability of ethanol in women versus men. In Phase II trials, glucuronidating enzymes and some efflux transporters have been shown to be more active in men than in women. Together with estrogens and androgen that alter transmembrane transporters, these processes contribute to efficacy of metabolism in both Phase I and II. Drugs used for treatment of cardiovascular disease, such as angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin II receptor blockers, diuretics, the aldosterone blocker eplerenone, antiplatelet agents, and oral antithrombotic medications, all show sex differences in efficacy and safety (325, 326). Over-the-counter nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and naproxen are more effective in men than women; there is more liver toxicity with acetaminophen use in women, whereas opioids and benzodiazepine work better in women. While some sex differences in metabolic clearance for statins and beta-blockers are known for these frequently prescribed drugs, dosing and adverse event monitoring in routine clinical practice is inadequate. Alosetron, a serotonin receptor 3 antagonist, is approved for treatment of severe irritable bowel syndrome–diarrhea symptoms in women, as it is largely ineffective in men (327). These findings emphasize that women and men take divergent routes (molecular mechanisms and signaling pathways) to reach the same destination (normal function or diseased state), with paths often intersecting. In the era of personalized medicine, there is no one-size-fits-all therapy, and considering sex-specific outcomes in pharmacokinetics and pharmacodynamics of drugs as well as clinical guidelines is warranted to ensure efficacy and safety of medications.

are apparent in all endocrine systems, which are modified by sex chromosomes and sex hormones, with temporal actions across the lifespan.

Blood Pressure Links Cardiovascular and Renal Diseases

Cardiovascular and renal diseases are linked by the relationship of each to arterial pressure (Fig. 5). The cardiovascular system determines arterial pressure, with the heart generating cardiac output and the blood vessels determining total peripheral resistance. The kidneys contribute by regulating extracellular and intravascular fluid volume, and hence blood volume, and venous return. It is established that CVD leads to chronic kidney disease (CKD) and that CKD leads to the development of CVD. For example, following a myocardial infarct, cardiac output declines and arterial pressure falls causing the kidney to vasoconstrict and retain extracellular fluid, with the effect to increase venous return and normalize cardiac output. However, this has the unwanted effect of placing further stress on the failing heart. Conversely, kidney failure causes fluid retention and hypertension (334). Thus, cardiovascular and kidney function are intertwined, as are the endocrine systems that regulate organ function; including the renin-angiotensin-aldosterone system

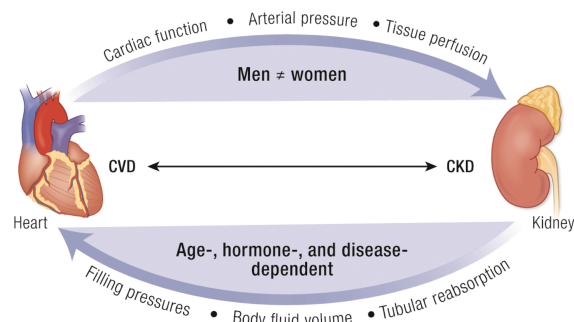


Figure 5. Heart and kidney functions are linked. Sex differences exist in many aspects of heart and kidney function at baseline and in CVD and CKD, as shown. Both organs feed-forward and influence each other's function. Genes, hormones, and age are some known factors that modulate this relationship in a sex-specific manner. Abbreviations: CKD, chronic kidney disease; CVD, cardiovascular disease.

(RAAS), the endothelin system, atrial natriuretic peptides, vasopressin, and glucocorticoid and mineralocorticoid hormones. There is an increasing recognition that there are fundamental sex differences in each of these systems. For example, aldosterone contributes to obesity-induced CVD with a greater impact in females than males (335). However, further research is required to fully elucidate the sex differences present in each endocrine system and how these impact disease development and progression.

Sex Differences in Arterial Pressure and Hypertension

Hypertension is a major risk factor for cardiovascular and renal disease. Over the lifespan there are age- and sex-related differences in arterial pressure. The majority of the data are derived from cross-sectional studies, but a few powerful studies have tracked arterial pressure over decades within a population (332, 336-339). Arterial pressure increases in both men and women with age, although the slope of the relationship is different between men and women. Sex differences in arterial pressure emerge during adolescence and are maintained throughout adulthood until women reach menopause (336, 337, 339). Arterial pressure is ~5 to 10 mmHg greater in men than age-matched women during the reproductive years (340-342). Postmenopause arterial pressure rises steeply in women regardless of race, ethnicity, or country of origin (340-342). One of the most striking characteristics of hypertension is that the prevalence and severity is lower in premenopausal women than in age-matched men. The prevalence of hypertension is ~10% in young premenopausal women, ~50% in postmenopausal women and by the age of 75 years almost ~80% of women are hypertensive (342-344).

Nonhuman mammalian species also display sex differences in arterial pressure. Arterial pressure in adult females is lower in normotensive dogs, sheep, rabbits, rats, and mice as compared with adult males (338, 345). Furthermore, in rodents, rabbits, and sheep, females of reproductive age are protected against the development of hypertension, such that arterial pressure increases significantly less in females than in males, in settings of disease (338). Thus, sex differences are present in the pathophysiology of cardiovascular and renal diseases. Yet, the mechanisms underlying the sexual dimorphism of arterial pressure in men and women as they age are poorly understood. However, extensive evidence indicates that sex hormones likely contribute to the regulation of arterial pressure through their actions on endocrine systems.

Sex Differences in Endocrine Control of Arterial Pressure and Kidney Function

There are subtle differences in most endocrine actions between men and women. It is not the maximal response of each system but rather the slope of the response that is altered. In this manner, a system responds maximally in a hemodynamic crisis (eg, hemorrhage) but in a sex-specific manner to lesser challenges. For example, a greater dose of the vasoconstrictor angiotensin II is required to increase arterial pressure in female than male mice (346). Consistent with this finding, the same dose of angiotensin II caused a

greater reduction in renal blood flow in men than women, with the suggestion that this was an angiotensin type 2 receptor (AT₂R) mediated effect (347). In rodents, females of reproductive age have a greater AT₂R to angiotensin type 1 receptor (AT₁R) ratio than males, which contributes to the reduced pressor response to angiotensin II (348). This has been indirectly demonstrated in women, in studies examining forearm vascular resistance responses to AT₂R blockade (349). The AT₂R also mediates a leftward shift in the pressure natriuresis-diuresis relationship, an effect that is greater in female than male mice (350). In women, indirect evidence also indicates a more pronounced role for the AT₂R in the regulation of renal blood flow responses to angiotensin II (347). This is linked to differential expression of components of the RAAS in males and females, which have been demonstrated in most mammalian species, including humans (351). In the context of the above example, estrogen interacts with the glucocorticoid response element on the X-linked *AGTR2* gene, to increase AT₂R expression in females (352). In addition, there are sex differences in human aminopeptidase A, aminopeptidase N, and angiotensin-converting enzyme 2 levels, responsible for generation of the angiotensin peptide fragments, angiotensin III, and angiotensin-(1-7), which have a high affinity for the vasodilatory AT₂R and Mas receptors, respectively (353-356). Lastly, there are marked and important sex differences in the production and function of aldosterone, although this has only recently been started to be examined (335). Thus, in females the RAAS is balanced toward the protective depressor RAAS arm, which at the lower physiological range may prevent arterial pressure increasing to the same extent as in males. However, this delicate balance may be lost in women after menopause and in the situation of metabolic syndrome.

Other vasoconstrictor systems also have sexually dimorphic actions. Endothelin-1 causes vasoconstriction via the endothelin type A receptor (ET_AR), and vasodilation and sodium excretion via the ET_BR. Testosterone increases ET_AR and estrogen increases ET_BR expression, which contributes to the differential control of arterial blood pressure and renal function between the sexes (357). Vasopressin, with important roles in circulatory and water homeostasis, is affected by age and sex. Urinary concentrating ability declines with age, but more steeply in women. Young men produce more concentrated urine than women, in part due to higher plasma arginine vasopressin levels and greater vasopressin type 2 receptor expression in the collecting ducts of the kidney in males (358, 359). Renal vasopressin type 2 receptor expression declines with age in association with a reduction in maximal urine concentrating ability (358, 359). Interestingly, aldosterone signaling via mineralocorticoid receptors is associated with increased CVD risk and is

enhanced in obese women (another example of how the RAAS is differentially modulated in females), which has been linked to leptin-induced endothelial dysfunction (360, 361). Moreover, evidence in rodents indicates that sodium reabsorption along the length of the renal tubule is sexually dimorphic, with reabsorption shifted to the later segments in females compared to males. This was associated with greater sodium epithelial channel expression, under the control of aldosterone, in the collecting duct, which could also contribute to the increased cardiovascular and renal risk associated with aldosterone in females (362). Finally, oxytocin, relaxin, and prolactin, which are traditionally known for their roles in pregnancy, have differential cardiovascular and renal actions in nonpregnant female and male rodents (348, 363, 364). Thus, evidence points to sex differences in endocrine control of extracellular fluid homeostasis and vascular function, which likely contribute to age- and sex-related disparities in renal and cardiovascular disease risk. Further studies are warranted to understand this complex issue more fully. In particular, it is important to take into account the subtle effects within the physiological range that counterbalance function of each hormonal system, rather than examine the impact of pharmacological doses which can mask sex differences in responses.

Cardioprotective Mechanisms in Women Sustain a Healthy Pregnancy

The cardioprotective mechanisms that predominate in women during the reproductive years enable the extensive hemodynamic adaptations required to meet the metabolic demands of the developing fetus and a successful pregnancy. During a normotensive pregnancy, blood volume increases and cardiac output increase by ~30% to 50%, but arterial pressure declines due to marked peripheral vasodilatation (365, 366). The associated renal vasodilatation accommodates an increase in glomerular filtration rate to process the additional blood volume, but an increase in vasopressin type 2 receptor expression enables increased tubule reabsorption of sodium and water. However, in women with preeclampsia, a pregnancy-induced form of hypertension, these cardiovascular adaptations are perturbed. Accumulating evidence now indicates that women with a history of pregnancy-associated hypertension have a 2- to 5-fold increased risk of CVD in later life (367). Understanding the mechanisms underpinning this dysregulation of vascular function in pregnancy-related hypertension may lead to the identification of new therapeutic targets for the treatment of cardiovascular disease in both sexes. For example, relaxin, which is known best for its role in pregnancy but is also produced in males, plays

roles in the regulation of renal function, blood pressure, and tissue fibrosis (363). Thus, it is a mistake to assign hormonal systems a specific role as most have wide-ranging tissue-specific pleiotropic effects.

Sex Hormones and Sex Chromosome Complement in CVD

Sex hormones contribute to sexual dimorphism in endocrine control of the cardiovascular system, with evidence suggesting that there is a “sweet spot” for both testosterone and estradiol, as unusually high or low levels of either promote disease (368-370). This has been the cause of apparent discrepancies in the literature. In particular, this remains a problem in animal studies in which the dose of estrogen used to study the impact of estrogen replacement in aged or gonadectomized models varies widely (~1000-fold), as does the route or length of administration; none of which accurately reflect the cyclic pattern of *in vivo* production. This lack of rigor into investigation of the effects of sex hormones in preclinical models likely contributes to the controversy that surrounds hormone replacement therapy for the prevention of CVD risk. Despite extensive evidence that hormone replacement therapy is cardioprotective, the negative results of the Women’s Health Initiative Trial effectively halted the use of hormone replacement therapy (371). Certainly, high-dose estrogen can increase blood pressure and cardiovascular risk in women (372). However, continued investigation supports the use of hormone replacement therapy in subsets of women, and further work in this area is required (373). In contrast, in men with low testosterone, beneficial cardiovascular effects are seen with testosterone replacement (374). In women with polycystic ovary syndrome, high testosterone levels are associated with elevated blood pressure (374). Dose-ranging studies are required to delineate these effects.

The sex chromosomes may have a direct impact on sex differences in the physiology and pathophysiology of the cardiovascular system and cardiovascular risk, independent of sex hormones. Human sex chromosome aneuploidies, such as Turner and Klinefelter syndromes, suggest that sex chromosome abnormalities can carry an increased risk of CVD. Women with Turner syndrome have around a 3-fold greater mortality and reduced life expectancy relative to the general population (375-377). CVD is a leading cause of increased mortality in Turner syndrome (375-377). Congenital cardiac anomalies, hypertension, coarctation of the aorta, diabetes, ischemic heart disease, and stroke are commonly associated with this condition (378). Similarly, men with Klinefelter syndrome have a high cardiovascular risk profile (379, 380), and an increased risk of

mortality from cardiovascular disease (381, 382). However, observations from studies in individuals with sex chromosome aneuploidies are complicated by confounding factors, including abnormal gonadal sex hormone levels associated with gonadal failure. Thus, it is very difficult to distinguish between hormonal versus genetic mechanisms and cardiovascular risk in these human conditions.

Experimental approaches, such as the FCG mouse model discussed in “Section I,” and Box 3 can discriminate between hormonal and sex chromosome effects in cardiovascular disease (115). Beyond genes on the sex chromosomes, there are sex differences in autosomal gene expression, which can be both organ or cell specific (383). In the kidney and the heart, hundreds of rat and human genes are regulated differently between the sexes (384–386). This disparate expression is triggered by sex hormones in ~30% of cases, with the other 70% linked to sex chromosome and microRNA dimorphisms (384, 385). For example, sex differences have been reported in the expression of nitric oxide synthase, tyrosine hydroxylase, and sodium channels in the rodent heart and kidney (332). However, few studies to date have compared gene expression and the effect on the proteome between the human sexes, and further studies are required.

Sex Differences in Pharmacotherapy for Cardiovascular and Renal Disease

Men and women respond to disease differently: kidney diseases progress faster in men than women, kidney transplants from women to men tend to fail more frequently than the reverse, and the effects of diabetes on the kidney differ between the sexes (387–392). Furthermore, symptoms and mechanisms of heart failure differ between the sexes (393). This suggests that sex-specific treatments for CKD and CVD could be required. There is currently little evidence to suggest that men and women respond differently to current treatments for hypertension (394). In large part, this is because clinical trials have lacked statistical power to take this into account. It will be difficult to achieve such an outcome for drugs that have already received FDA approval. However, some treatments are more frequently prescribed, without any basis in evidence (395). There are also marked differences in pharmacokinetics and pharmacodynamics (see Box 4), leading to more frequent adverse drug reactions in women, related to differences in drug clearance and breakdown (396). Therefore, sex should be taken in account for new treatments seeking approval in the future. When women are considered, important and unexpected sex differences are observed in almost every aspect of cardiovascular and renal function in health and

disease. Further research is required to fully understand these differences, and in turn to guide the development of sex-specific treatment guidelines for CVD and CKD.

Section V

Challenges for the Future of Sex Differences Research—Areas Requiring Special Attention

Sex differences exist in anatomy, behavior, and physiology across the animal taxa. By extension, because of these innate differences, sex differences exist at molecular and cellular levels in mechanisms that underlie these processes. Despite concerted efforts by the Office of Research on Women’s Health and the Organization for the Study of Sex Differences in educating researchers about the distinction between sex versus gender, the indiscriminate use of the word “gender” continues to pervade scientific literature. The sex of established cultured cell lines is another issue; in addition to aneuploidy, chromosomal numbers change as cells are passaged and are dependent upon the tissue of origin (397, 398), but this aspect is beyond the scope of this Statement. Not surprisingly, sex differences are seen in etiology, prevalence, and outcomes in a myriad of human diseases that range from psychological and autoimmune to gastrointestinal, cardiovascular, renal, and reproductive; SARS-CoV-2 causes more severe COVID-19 disease in men than in women despite similar infection rates (399–401). Besides genetic makeup (predisposition), extraneous factors, such as the socioeconomics, demographics, education level, profession, age, and the environment, greatly influence an individual’s health; COVID-19 disease outcomes especially highlight the contribution of these extraneous factors in health disparities. Factors such as the endocrine-disruptive chemicals can disproportionately affect one sex over the other; regardless, whether favorable or adverse effects are present in one or both sexes, the effects would impact trans and cisgender persons, and hence these sex-specific effects should not be overlooked or underestimated (402). Some human studies addressing sex differences take these factors into account, whereas others are more selective. Many studies of disease pathways are sensitive to levels of gonadal steroid hormones, which contribute to sex differences. In human studies, unless gender information is explicitly collected or available, the study deals with biological sex, not gender. Use of sex and gender interchangeably deemphasizes the importance of studying gender as an independent variable.

In animals or experimental models of human diseases, effects of estrogens have been investigated more often than effects of progestins and androgens, which should

be corrected. Paradoxically, female sex is often excluded from experimental design on the basis that: (i) the estrus cycle will interfere with data interpretation; (ii) mechanisms that operate in the male sex will operate in the female sex and thus only need to be confirmed in females; (iii) metabolic demands are similar between the sexes; (iv) the X chromosome in males and females is subject to similar regulation; and (v) autosomal genes will be subject to equal variance between the sexes. The same studies often ignore the diurnal cycling nature of testosterone in males; testosterone levels in male rodents can show more day-to-day variability than estrogen and progesterone levels in females. Other steroid hormones, such as glucocorticoids, that show circadian rhythm and whose levels differ between the sexes also influence gene expression and function. In rodents but not primates, sex differences in secretion of growth hormone result in sexually dimorphic hepatic metabolism of drugs and xenobiotics (403). In rodents, endocrine disruption can have transgenerational effects on male and female reproductive systems (404). Since changes in hormone levels and gene expression are dynamic, can be localized, and are spatiotemporally distinct, no one study design or condition can be used as a gold standard. Animal housing and handling conditions can also create sex differences, and thus any experimental design and data interpretation should take these variables into account. If sex-segregated data does not differ for the aspects under study, then data can be pooled from the 2 sexes and reported accordingly.

Studies in animal models have just begun to uncover unequal effects of the sex chromosomes in XX vs XY cells, so we expect further discoveries about such effects in the future. Once genes that cause sex differences are discovered in animals, the findings generate new hypotheses and rationalize human studies to determine whether the same gene also creates sex differences in humans. That question can be studied by the methods of human genetics, relating genetic variation to disease incidence and outcome. Without the animal studies, however, it is difficult to understand detailed molecular mechanisms. It is also important to remember that no single rodent or animal model can capture the complexity of any human disease, but each model provides valuable insights into one or another major aspect of disease. If different etiologies of a given disease share mechanisms, then mimicking the precise conditions that initiate human disease may not be critical.

The study of sex chromosome effects is in its infancy and has focused on proving that sex chromosomes play a role and finding the genes responsible for the effects. So far there has been little effort to understand how these factors interact with steroid hormones to cause sex differences. If

both types of factors cause differences in disease incidence, are they affecting the same or different downstream pathways? Do their effects converge, or do they independently affect different mechanisms that each influence a complex disease? Do male-biased factors (hormones, Y-chromosome genes) act synergistically to induce a male-specific state, or do they counteract each other to reduce the difference between males and females (123, 405)? Are the diverse sex-biasing factors changing in their effects across the lifespan, leading to changes in the type or amount of sex difference at different ages?

When studying sex differences in animal models of human diseases, it is important to first understand and elucidate differences at baseline in gonadally intact animals. As pointed out earlier, steroidogenic enzymes are also present in nongonadal tissues, especially the brain, thus it is not entirely possible to eliminate effects of sex steroids from all tissues. Moreover, tamoxifen-inducible *Cre* recombinase used to routinely perform lineage tracing and gene inactivation studies in mice has its own problems (406, 407) that are largely ignored and can further confound sex-specific data analysis; tamoxifen antagonizes actions of estrogen receptor- β and inhibits expression of over 70 genes (408), but the contribution of these tamoxifen-regulated genes on study results and outcomes is never accounted for and requires careful consideration. Before mechanisms behind sex differences in physiology and disease can be elucidated, a fundamental understanding of sex differences that exist at baseline, is needed.

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