How to Study the Origins of Sex Differences in Brain and Behavior

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Introduction

This year marks the 48th Annual Meeting of the Society for Neuroscience. It is also almost 60 years since the discipline of neuroendocrinology was born, after seminal papers established that gonadal hormones during fetal development exert enduring changes on the brain, which then determine adult reproductive behavioral and physiological phenotypes (Phoenix et al., 1959). During this time span, the fields of neuroscience and neuroendocrinology have lived largely parallel lives, having insufficient influence on each other. This has contributed to the situation we have today, in which neuroscience has advanced almost exclusively by studying the brains of male animals, and neuroendocrinology has largely emphasized endocrinology at the expense of neuroscience. But today, all that is changing. Part of the change involves making more accessible and appealing the study of sex differences in the nervous system by (1) demystifying females (they are not just fluctuating hormones) and (2) conveying the heuristic power of contrasting fundamental neuroscience processes in males and females. Toward that end, I will present some fundamentals.

Sex Determination, Sexual Differentiation, and Sex Differences

Sex determination begins with genetics and the sex chromosomes. All mammals and birds, some insects, most fish, and a smattering of amphibians and reptiles are sex-determined as a function of specialized chromosomes that differ between males and females. In mammals, XX and XY chromosomes determine male and female, and the same goes for *Drosophila*. But in birds, females are WZ while males are WW. In mammals, it is a single gene on the Y chromosome, *Sry*, that directs the bipotential gonadal anlage to differentiate into a testis (Goodfellow and Lovell-Badge, 1993). If that gene is missing or mutated, or if there are two X chromosomes, the gonadal precursor will develop into an ovary. Formation of a testis occurs extremely early in development, when the brain is still a gelatinous mass, and becomes an active endocrine organ shortly thereafter, synthesizing hormones that repress the survival of the female reproductive tract (e.g., uterus, cervix, vagina) and promote the survival of the male reproductive tract (vas deferens, seminal vesicles, etc.). By the second trimester in humans, and during the third week of pregnancy in rodents, the testis of a male fetus is synthesizing close to adult levels of androgens (Fig. 1). It is this phase of

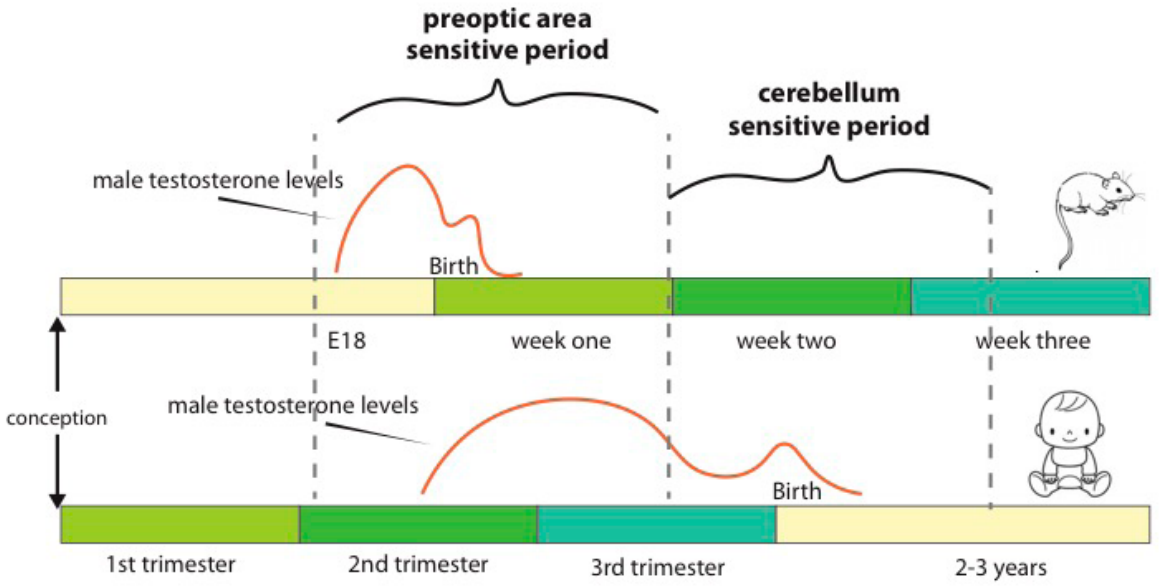

Figure 1. Sensitive periods at different life stages of rats and humans. The developmental profile of the rat is shifted from that of humans, in that a newborn pup is roughly equivalent to a mid- to late-gestation human fetus. The sensitive period for sexual differentiation of the preoptic area in the rat is operationally defined by the onset of testicular androgen production in male fetuses on E18 and the loss of sensitivity of females to exogenous hormone treatment by the end of PN week 1. In humans, the sensitive period for the preoptic area begins during the second trimester with fetal androgen production and probably ends before birth (although this conclusion is constrained by a lack of experimental data). The sensitive period we have identified in cerebellar development occurs during PN week 2 in the rat, which corresponds to the peripartum period in the human. Factors constraining the sensitive period in the rat are the onset and offset of gene-expression profiles. Whether a similar profile exists in humans is currently unknown. Reprinted with permission from McCarthy and Wright (2017), Fig. 4. Copyright 2017, Elsevier.

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steroid production that drives sexual differentiation of the brain by simultaneously initiating multiple distinct cellular and molecular process that can be collectively referred to as "masculinization." The purpose of masculinization is to impart anatomical and physiological changes that will ensure that, later on, the brain residing in the male-determined body will support spermatogenesis, motivation to mate with females, territorial defense and/or competition or aggression against other males, and so on. The result of sexual differentiation of the brain is sex differences. However, brain sex differences can be achieved in other ways or even reversed, as will be discussed below.

Critical versus sensitive periods

The formation of the brain proceeds in epochs. Some of these are intrinsic programs, and others are periods of sensitivity to stimuli, either internal or external, such as the need for light for the proper formation of the visual system. These are called "critical periods" because the exposure must occur during a specific developmental epoch or the window of opportunity is forever lost. Sensitive periods are distinct from critical periods; they are times when a perturbance will have an enduring effect that would not occur if the exposure happened at another time. *In utero* inflammation and the risk of autism spectrum disorders or schizophrenia in the offspring are emerging examples of sensitive periods and their consequences. The sexual differentiation of the brain is unique in that it consists of a critical period for masculinization: exposure to androgen must occur during that developmental window and therefore exists in only one sex (Fig. 2). But there is also a sensitive period for females when exposure to exogenous androgens can induce masculinization, if only during a specified period (Fig. 3). In our rodent animal models, this period extends into postnatal (PN) week 1; thus, treating

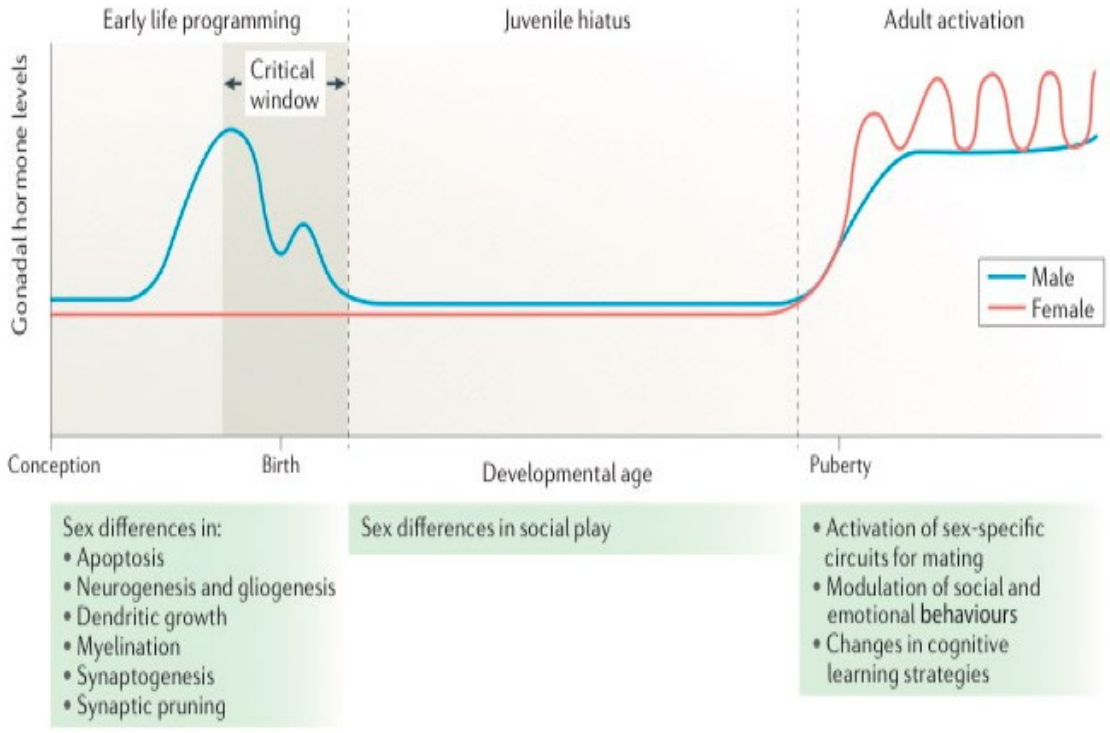

Figure 2. Early life programming of adult sex differences. Steroidogenesis by the perinatal male testes results in elevated circulating testosterone, which is aromatized to estrogens in the brain. Combined androgen and estrogen action modifies multiple developmental process throughout the brain in a regionally specific way during a narrow, sensitive time window. These processes include cell genesis, neuronal migration, dendritic growth, synaptogenesis, and synaptic pruning and cell death, among others. Within a few days of birth, the elevated steroids in males decline to undetectable and are equivalent to the female's. Both remain there during the juvenile hiatus, a time of heightened rough-and-tumble play behavior by males. After puberty, both sexes reestablish gonadal steroidogenesis that is dimorphic in amount and patterning. This hormonal milieu acts upon the neural substrate that was organized early in life to promote the expression of sex-typic physiology and behavior, the most obvious of which is copulatory. Reprinted with permission from McCarthy et al. (2017), Fig. 1. Copyright 2017, Nature Publishing Group.

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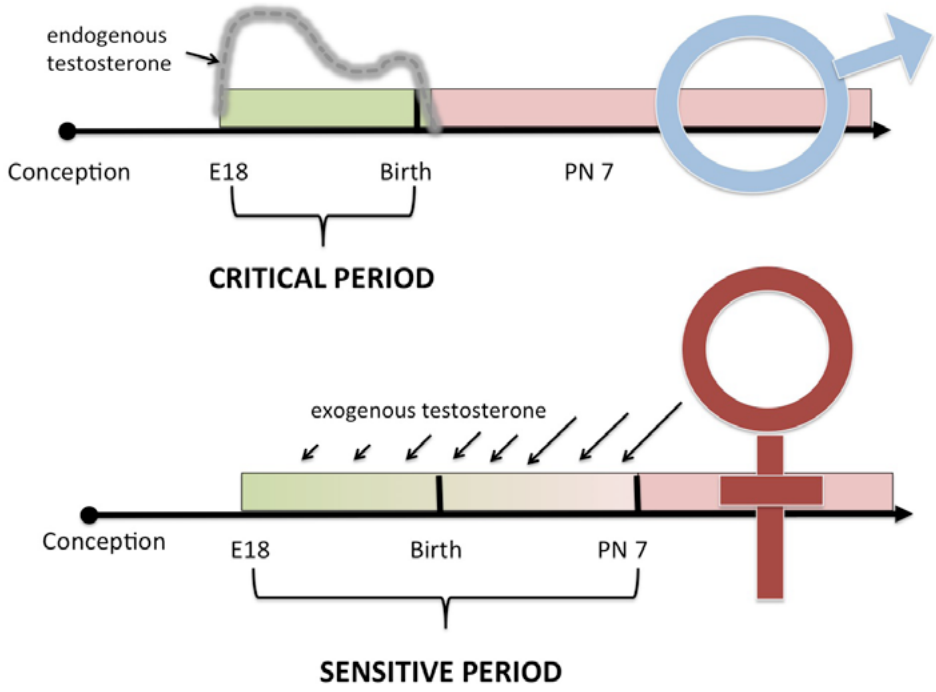

Figure 3. The critical and sensitive periods for sexual differentiation. Masculinization of the brain occurs during a critical period that begins with the onset of endogenous testosterone production from the fetal testis on E16 (mouse) or E18 (rat). Circulating testosterone levels fall within hours of birth, and the critical period ends shortly thereafter as the process of masculinization irrevocably proceeds. Females are not exposed to endogenous testosterone as the ovaries are quiescent; therefore, gonadally derived hormone exposure is limited to testosterone exposure from their littermates. Females also remain sensitive to exogenous testosterone treatment for up to 1 week after birth, with increasingly larger doses (indicated by larger arrows) required as sensitivity wanes. After 7–10 days, the process of feminization irrevocably proceeds. Because of the unique synthesis of testosterone in males but the shared sensitivity of both sexes to this steroid hormone, males have a short critical period whereas females have a longer sensitive period. The ability to sex-reverse females postnatally with exogenous testosterone provides a highly useful but imperfect tool for the study of sexual differentiation. Reprinted with permission from McCarthy et al. (2018), Fig. 2. Copyright 2018, Elsevier.

females with testosterone or its aromatized product, estradiol, will induce masculinization if the dose is high enough. This is a highly useful experimental tool for studying the process of masculinization because, in males, it begins *in utero*, and once begun is very difficult to block (McCarthy et al., 2018). This does create a conundrum, though, in that females treated with exogenous hormone to study masculinization are several days older than their male siblings that underwent normal masculinization. In rodents, every day counts, particularly in neonates, but there is no easy way around this confound.

Androgens versus estrogens and the "aromatization hypothesis"

The shift in timing of the critical period in males versus the sensitive period in females is just one of the many challenges associated with studying sexual differentiation and deciding how to design and interpret experiments appropriately. Early in the days of defining the parameters mediating sexual differentiation, scientists recognized the need to use negative controls for the treatment of females with testosterone to be sure they were not observing some nonspecific effect of treatment that masqueraded as masculinization. Cholesterol was one obvious negative control since it carries all the same properties as steroid hormones but does not bind to steroid receptors. But estradiol was considered an even better control because it is a potently active steroid in its own right, and generally considered a female hormone, but does not activate androgen receptors. To the investigators' surprise, estradiol proved to be an even more effective inducer of masculinization when given to females. More importantly, blocking estrogen activity in newborn males, either with an estrogen receptor antagonist or inhibitors of aromatase, effectively blocked masculinization in males. These effects were formulated as the "aromatization hypothesis," which is now established fact and incorporates several properties that are essential for understanding sexual differentiation of the brain (McEwen et al., 1977).

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The first property is that testosterone is a precursor (or prohormone) that is aromatized to estradiol in a rate-limiting step by the enzyme Cyp19a, or aromatase (Fig. 4). The second is that neurons in the brain express the aromatase enzyme and thereby locally synthesize estradiol from the androgen precursors in circulation synthesized by the fetal testis. The distribution of aromatase expression is not random, but is concentrated in specific nuclei and regions, with levels varying between brain areas and the sexes (Fig. 4). The distribution of estrogen receptor(s) expression and aromatase is overlapping but not identical, and the degree to which steroid is synthesized in one cell and released to act on an adjacent cell is not entirely clear but certainly plausible. The third property is that estrogens from the maternal circulation must be excluded from gaining access to the fetal brain, or all the pups will be masculinized. This conundrum is solved by the nifty trick of a steroid-binding globulin called alpha-fetoprotein, found at very high levels in the fetal circulation, which binds estrogens but not androgens. Alpha-fetoprotein creates a sponge, or trap, in the fetal circulatory system exclusive to estrogens but allows androgens to gain access to neurons and be locally converted to estrogens. How precisely androgens access the interior of neurons or other brain cells is not known, but there is reason to believe it is a somewhat regulated process and may play an important but undetermined role. There is also lingering evidence that alpha-fetoprotein may itself be a signaling molecule and may even deliver estrogens to specific cells (McCarthy, 2008). Finally, not all masculinized endpoints are the result of estrogen action; some are regulated directly by androgens. The most well characterized endpoints are the number of motor neurons in specific spinal cord nuclei and the number of cells in the amygdala (Morris et al., 2004). Still other endpoints appear to involve both estrogen and androgen action (Waddell et al., 2013).

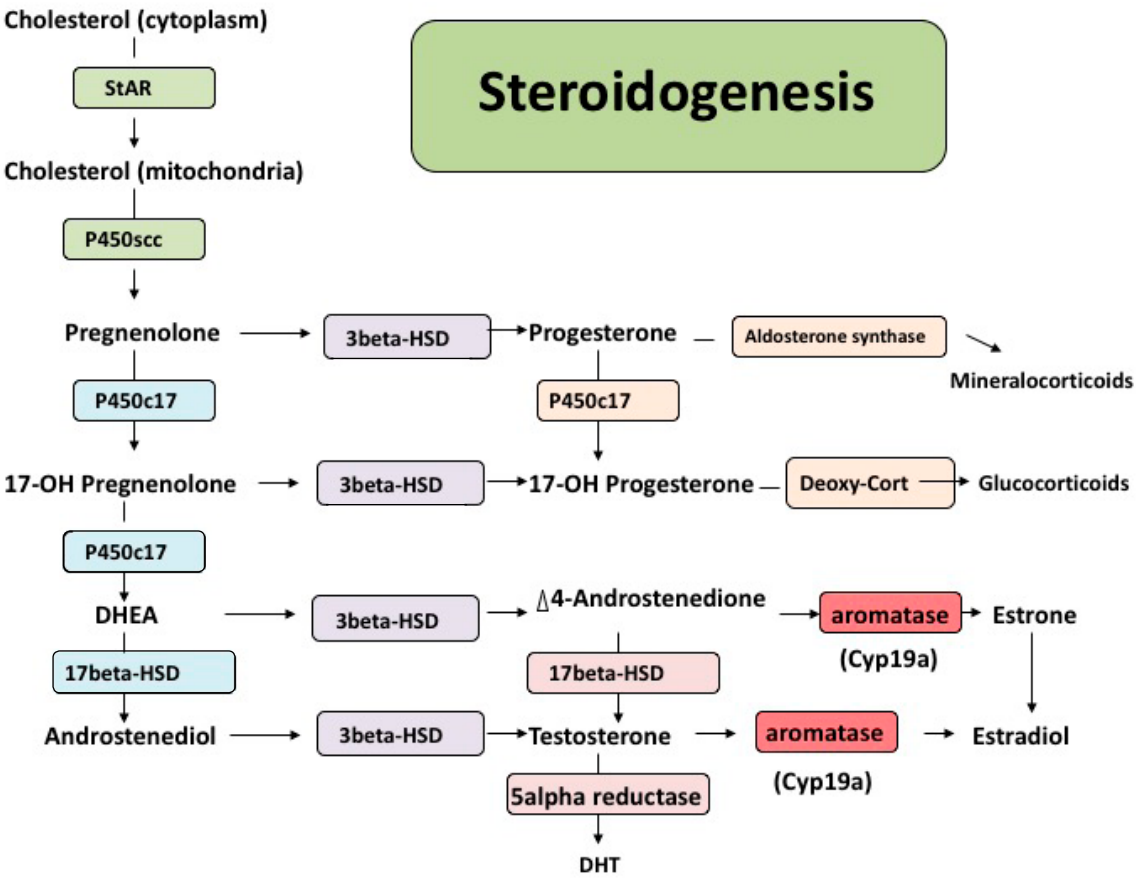

Figure 4. Steroidogenesis. All steroids begin life as cholesterol and, through a series of enzymatic reactions that mostly remove hydroxyl groups and carbons, become either progestins, glucocorticoids, mineralocorticoids, androgens, or estrogens. Testosterone is a precursor to estradiol, which requires aromatization by the enzyme Cyp19a. DHT is also an enzymatic by-product of testosterone following 5-alpha reduction; however, it cannot be converted into estradiol and is therefore called a nonaromatizable androgen. Deoxy-Cort, deoxycorticosterone; DHEA, dehydroepiandrosterone; HSD, hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein.

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Experimental Approaches

How to determine which steroid is driving sexual dimorphism

There are two general approaches to the question of whether a particular endpoint is sexually differentiated by estrogens, androgens, or some combination thereof. The first is the historically more recent approach of using genetically modified mice, which have proven highly effective at confirming the central role of the alpha form of estrogen receptor 1 (Ers1) to masculinization (Ogawa et al., 2000) and revealed an unexpected role for the beta form in defeminization (Kudwa et al., 2005). Mice lacking the aromatase enzyme or alpha-fetoprotein further confirm the centrality of estradiol to most hormonally mediated differentiation events (Bakker et al., 2003, 2006), whereas the androgen receptor–deficient mouse highlights the importance of testosterone and other androgens (MacLusky, 1988).

The genetic mutability of the mouse is a powerful tool, but a fundamental and unavoidable shortcoming is that once the gene is inactivated in development, it remains so for life, making it impossible to differentiate developmental (often called "organizational") effects from those exerted in adulthood (referred to as "activational"). So the second approach for investigating sexually differentiated endpoints is the use of steroids. Fortunately, steroids are fairly easy to administer exogenously, can be administered as early as the day of birth, and are structurally the same across all species. That said, steroids generally do not follow lawful dose-response curves, and their action is complicated by the need for cofactors, nuclear as well as membrane receptors, rapid and enduring effects, and much more that is beyond the scope of this discussion. However, one does not need to be expert in endocrinology to properly administer hormones during development, and some general guidelines follow.

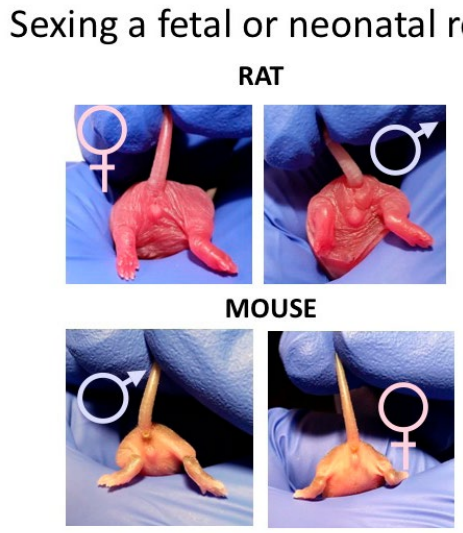

Figure 5. Sexing rat and mouse pups. The anogenital distance is longer in males than in females and can be either measured with calipers or assessed visually with experience. Moreover, the male usually has a slight swelling and sometimes pigmentation in the anogenital area, indications of the future scrotum.

Sexing newborn rodent pups

Distinguishing male and female pups from each other is a simple task for the trained eye but a daunting one for those not familiar with working with such small animals (newborn mice and rat pups weigh on the order of a few grams). Even my institution's animal care and use committee once returned my proposal with the question, "How will you tell the boys from the girls?" I restrained myself from replying that it was easy—the boys are blue and the girls are pink—and instead explained how one could simply look at the genitalia and distinguish male from female with 99.9% accuracy. As shown in Figure 5, males have a longer distance between the anus and the urethra, and there is a slight swelling (future scrotum), often accompanied by some pigmentation.

Although this approach is easily used on animals at the time of birth and can be effective for very late-stage embryos, dissection of the abdomen to identify the presence of testes provides stronger assurance. The testes of male rodents do not descend until well after birth and can be readily found in the lower body cavity, where they appear as two small pearly grains of rice, one on each side. The female ovary can also be seen but is much more difficult to detect, and therefore less reliable.

The testes develop very early in pregnancy, but the further one goes back toward conception, the more difficult they are to see. However, sex can also be confirmed genetically using PCR for Y-chromosome-specific genes, and this is an excellent solution when immediate identification is not required. It is tempting to think that one should conduct PCR for *Sry*, but this is actually not ideal, as there can be multiple copies of this gene in some species, with only one being functional. Instead, it is recommended to measure the gene *Jared*, which is found on both the X and Y chromosome but is smaller on the Y chromosome, therefore producing two PCR bands in males but only one in females (Clapcote and Roder, 2005). An alternative approach is to measure repetitive sequences on the Y chromosome as a proxy for *Sry* (Itoh et al., 2015).

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Treatment with exogenous steroids

Determining whether a sexually differentiated endpoint is mediated by androgens, estrogens, or some combination is fairly simple to achieve by exogenous treatment with steroids, but there are some caveats. As noted earlier, estrogens are sequestered in the fetal blood by alpha-fetoprotein; thus, an exogenous dose must overwhelm the binding capacity of this steroid-binding globulin. We find that in rat pups, a dose of 100 μg estradiol benzoate given subcutaneously in a sesame oil vehicle once on the day of birth and again the next day fully recapitulates any estrogen-mediated sexual differentiation processes. Although this dose is very large compared with the 10 μg used to induce sexual receptivity in an adult (and so tends to alarm reviewers on occasion), we have confirmed that it increases brain levels of estradiol in females only to the level of males in the most sexually dimorphic region: the preoptic area. This finding indicates that we are inducing physiological levels in the CNS (Amateau et al., 2004).

Treatment of a newborn pup with estradiol is an effective approach, but some argue it circumvents the normal process of aromatization and precludes any additional or supplementary effects that might involve direct androgen action. Thus, if you desire to be fully confident that an effect is mediated solely by estradiol, additional groups should include testosterone treatment, in which you expect to see the same endpoints, and dihydrotestosterone (DHT) treatment (the latter is a nonaromatizable androgen and therefore activates only the androgen receptor). To compare across groups, we keep the doses of each steroid the same. In order to ensure the steroid is long lasting in the circulation, we use estradiol benzoate, testosterone proprionate, and DHT proprionate. The benzoate and proprionate moieties do not impact steroid action but slow the release from the oil depot and muscle, thereby extending the half-life and more closely mimicking endogenous steroids.

When treating neonates, the preferred vehicle is sesame oil and the preferred injection volume is 0.1 cc given subcutaneously. We use a 30 ga needle and inject under the skin at the scruff of the neck or over the rear haunches. To prevent the oil from flowing back out the injection site, the skin is pinched between thumb and forefinger as the needle is withdrawn and then the site is dabbed with either New Skin or Super Glue to seal the hole. This step is essential, as steroids are highly lipophilic: if the oil escapes, there will be an easy transfer of steroid to littermates via absorption through the skin.

We generally inject pups without anesthesia, but they can be cryoanesthetized if desired. This is a good practice when first learning the technique, as it eliminates the squirm factor. Cryoanesthesia is a fancy word for chilling. Pups are placed on top of tin foil over ice in a standard lab ice bucket and then placed in a 4°C refrigerator for 10–20 min, depending on age. When the pups are blue and motionless, they are anesthetized. Do not place multiple pups together, as they will huddle for warmth. Recovery from anesthesia is achieved by placing them under a mild heat lamp (or just a light bulb) or on a very low heating pad. More pups die from being overheated than overchilled, so be careful, although it is possible to overchill as well. Only when pups are pink and wiggly should they be returned to the dam.

Antagonizing or blocking endogenous steroids

Treating with exogenous steroids is easy, but blocking endogenous steroids is not. The difficulties arise from the timing of administration, effectiveness, solubility, and specificity.

Timing

The goal of blocking endogenous steroids is often to interfere with or prevent naturally occurring masculinization. But this process begins *in utero* with the surge in androgen production around embryonic day (E) 16 through E18. Blocking steroid synthesis in the pregnant dam would compromise the pregnancy, and it is not technically feasible to selectively treat the developing fetuses. The critical period for males does extend postnatally, but elevated steroid levels last for only ~2 hours after birth, which essentially means one must observe pups being born and treat them within an hour or so. Anyone who works with pregnant rats and mice appreciates that this is easier said than done, as they do not commence delivery on command or to suit your work schedule. However, not all actions of endogenous steroids appear to be restricted to those first few hours. We have antagonized estrogen production and receptor action in the hippocampus of neonates on the day of birth and one day later to good effect (Bowers et al., 2010).

Effectiveness

Highly effective aromatase inhibitors have been available for some time because of their potential therapeutic use against metastatic estrogen-dependent breast cancer (Blakemore and Naftolin, 2016). Aromatase inhibitors come in two types. The first are analogs of the androgen precursors that bind irreversibly to the enzyme. Formestane is

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the most commonly used inhibitor of this type and is beneficial for its high solubility in biologically tolerable vehicles, such as sesame oil. It can readily cross the blood-brain barrier (BBB), but it is only ~75–80% effective at reducing estradiol production. Given the need for complete inhibition in cancer treatment, this led to the development of the second type: nonsteroidal aromatase inhibitors. The most effective one is letrozole, which has ~99% effectiveness. However, it is difficult to dissolve in standard vehicles and does not reliably cross the BBB. For this reason, we have relied on Formestane (100 μg), which we find reduces brain estradiol in a neonate to near undetectable levels (Konkle and McCarthy, 2011).

Specificity

The most commonly used estrogen receptor antagonist is tamoxifen, which has advantages in terms of solubility and penetrance of the BBB. We have found tamoxifen to be an effective blocker of endogenous estrogen actions when given at a dose of 100 μg (notice the pattern here, as mentioned above: there is not a strong dose-response curve with steroids). The concern with tamoxifen is its well-known capacity to occasionally act as an agonist, not an antagonist. However, its agonist effects seem to be restricted to its actions in bone or after an extended period of exposure. A purer antagonist is ICI 182,780 (Fulvestrant), though its shortcoming is poor penetration of the BBB, so it is a trade-off. Both tamoxifen and ICI have affinity for the alpha and beta forms of the estrogen receptor, and thus cannot be used to distinguish between these isoforms. If one needs to distinguish between ERα and ERβ (and I encourage anyone to ask themselves first whether the distinction is really important), then there are relatively specific agonists and some more recently developed ones that can be employed. However, the limitations of pharmacology are in strong evidence here. If one is working with mice, it may be far better to use a genetic approach.

As difficult as antagonizing estrogens is, blocking androgens is even messier. First, selectively blocking synthesis is more challenging, as this class of steroids is further up the chain of steroidogenesis (Fig. 4) and the synthetic enzymes are less specific than is the obligatory aromatase for estrogen synthesis. The most commonly used androgen synthesis inhibitors target the 17-a lyase (CYP17) enzyme, but these have many off-target effects, and often, residual androgen receptor activity (Stein et al., 2014). In general, my recommendation is to avoid trying to inhibit androgen synthesis as an experimental approach.

Second, the only readily available androgen receptor antagonist is flutamide, and it is a lousy one at that. We have consistently found (and been told anecdotally by others) that flutamide is an effective androgen receptor antagonist only when used to block exogenous androgen action. Using flutamide to block endogenous steroid simply doesn't work. Nonetheless, it can be useful for confirming an androgen effect by giving testosterone to females with or without flutamide with the assumption that flutamide will effectively block any effects of testosterone.

Masculinization, Feminization, and Defeminization

Up to this point, we have been emphasizing the process of masculinization because it is the process by which the male is sexually differentiated from the female, which is the default. In other words, feminization of both brain and body is the developmental trajectory that occurs in the absence of testis and androgen production. That is, an ovary is not required for female development, although it is essential to adult reproductive capacity. This does not mean that feminization of the brain is not an active process (it surely is), but it is much harder to discern what it is in the absence of some third "neither male or female" phenotype. There is evidence of a later critical period in female brain development (during PN week 2) that may involve estrogen production by the ovaries (Bakker and Baum, 2008), but unfortunately, this concept is not fully developed. Perhaps the best angle from which to discern feminization is its polar opposite: defeminization, an active process whereby the female phenotype is removed. This phenomenon is best illustrated (and perhaps limited to) the sex-typic mating behavior seen in rats and mice, wherein males show mounting behavior toward sexually receptive females, which respond with lordosis, a posture that allows the male to intromit his penis. Because feminization is the default, the neural circuitry of lordosis comes as "preinstalled software." Removing that programming is achieved by defeminization, which is also driven by androgens aromatized to estrogens in developing males but via distinct cellular mechanisms (Schwarz and McCarthy, 2008). Why such a system has evolved is a mystery, and whether it applies outside the context of sex behavior is debatable. However, it does tell us that multiple independent processes occur simultaneously in the developing brain that ensure as little overlap as possible between males and females in certain key reproductive functions. Notably, no parallel process of demasculinization exists in females, and when masculinization is blocked in

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males, it is best referred to as "dysmasculinization," since it is the disruption of a normal process rather than a normal process itself.

Nonhormonal and Nongenomic Factors: The Environment

The advent of the "four core genotypes" mouse model described elsewhere in this course has irrefutably demonstrated the impact of sex chromosome complement on brain and behavior. Given that genetics is a constant, and is not exclusive to development, we will not review it further here. However, any discussion of sex differences must consider nonhormonal and nongenomic factors: i.e., the environment. In humans, the gender of a child affects everything, including the way it is dressed, handled, and even spoken to, all of which occurs during major epochs of brain development. Even in rodents, the maternal dam interacts with her male and female pups differently. Beginning with anogenital licking and grooming (which is essential to pup survival, as they cannot urinate or defecate on their own), the dam performs this function on male pups more frequently than on female pups. The extra attention the males receive provides vital stimulation to the developing motor neurons that innervate the penis, promoting myelination and ultimately enhancing adult reproductive functioning (Moore, 1984). Males also receive preferential treatment if separated from the nest. For very young pups that cannot see, locomote, or thermoregulate, being isolated from the dam is an alarming circumstance to which they respond with vigorous and frequent ultrasonic vocalizations. Interestingly, the males are more frequent and more vigorous in their distress calls, and this motivates the dam to retrieve them back to the nest more quickly than she does the females (Bowers et al., 2013). Although not directly tested, this rapid response can be inferred to result in less stress to the male pups after a separation.

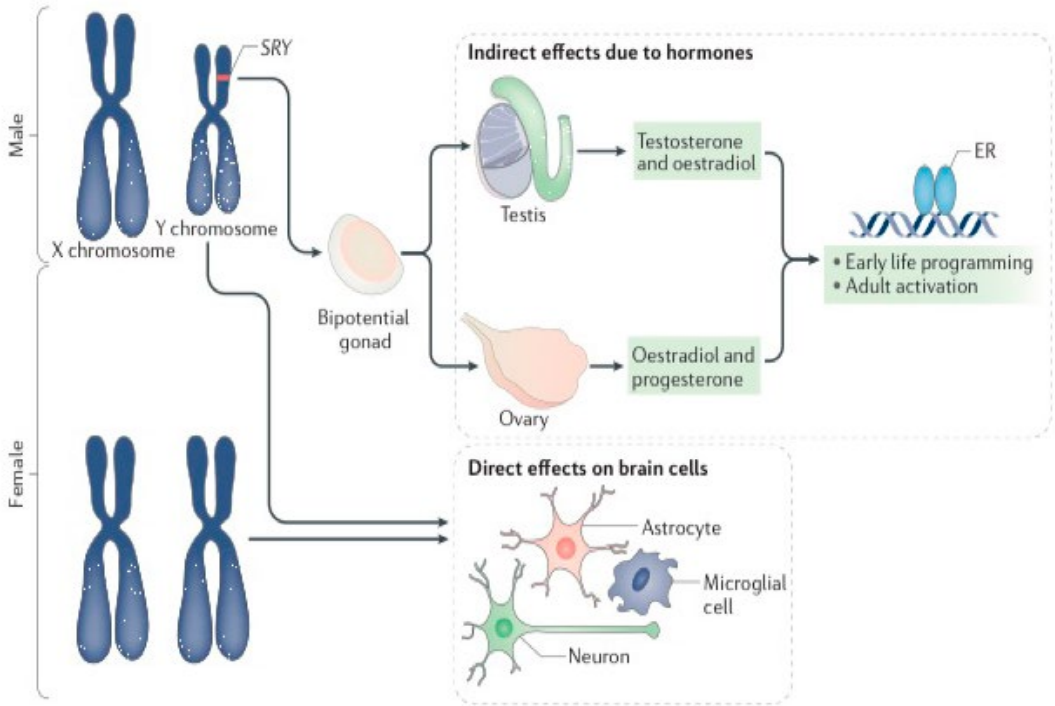

Figure 6. Epigenetic sources of brain sex differences. Several critical epigenetic mediators are X and Y linked, such as the histone lysine demethylases Kdm6a and Kdm5c, which escape X inactivation in the brain. The Y-linked homologue to kdm6a (UTX), kdm6c (UTY), is expressed at higher levels in the male brain (Xu et al., 2008). Sex-specific expression of epigenetic modifiers such as these has the potential to establish widespread sex differences in the chromatin landscape, gene expression, and thus structural and functional sex differences in the brain. X-linked chromatin-binding proteins such as MeCP2 have also been shown to be important for establishing brain sex differences. Male gonadal hormones reduce the expression of the methyl-binding protein MeCP2 in the amygdala. They have also been shown to reduce DNA methyltransferase activity and methylation genomewide in the preoptic area and alter methylation on specific promoters related to brain masculinization, such as the estrogen receptors (ERs) and progesterone receptors. Hormonal modulation at the level of histone methylation and acetylation has also been demonstrated in the preoptic area and bed nucleus of the stria terminalis, potentially mediating both activational and repressive chromatin states. Reprinted with permission from McCarthy et al. (2017), Fig. 2. Copyright 2017, Nature Publishing Group.

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The final issue I want to raise is that, even *in utero*, male and female fetuses can respond profoundly differently to stress that occurs to the pregnant dam, and this can have life-long consequences. Stress as early as the first week of pregnancy impacts both the placenta and the brain of male pups, but has seemingly no effect on the females (Howerton et al., 2013). Effects on males can be so profound that they persist to the next generation via epigenetic modifications (Morgan and Bale, 2011). Indeed, epigenetic changes occurring during gestation, or perinatally during hormone-mediated sexual differentiation, are likely the foundation on which sex differences persist following either perturbed or normal development (McCarthy and Wright, 2017) (Fig. 6).

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