ARTICLE

Sexual Differentiation of the Brain in Man and Animals: Of Relevance to Klinefelter Syndrome?

MARGARET M. McCarthy*

The developing brain is highly sensitive to the organizing effects of steroids of gonadal origin in a process referred to as sexual differentiation. Early hormone effects prime the brain for adult sensitivity to the appropriate hormonal milieu, maximizing reproductive fitness via coordinated physiology and behavior. Animal models, in particular rodents, have provided insight into general principles and the cellular and molecular mechanisms of brain differentiation. Cellular endpoints influenced by steroids in the developing brain include neurogenesis, migration, apoptosis, dendritic growth, and synaptic patterning. Important roles for prostaglandins, endocanabinoids, and epigenetics are among the many cellular mediators of hormonal organization. Transference of general principles of brain sexual differentiation to humans relies on observations of individuals with genetic anomalies that either increase or decrease hormone exposure and sensitivity. The physiology and behavior of individuals with XXY (Klinefelter syndrome) has not been considered in the context of sexual differentiation of the brain, most likely due to the delay in diagnoses and highly variable presentation. The behavioral phenotype and impairments in the domains of speech and language that are characteristic of individuals with XXY is consistent with the reduced androgen production associated with the syndrome. Hormone replacement appears effective in restoring some deficits and impact may be further improved by increased understanding of the hormonally mediated sexual differentiation of the brain.

KEY WORDS: neurogenesis; androgen; estrogen; preoptic area; hippocampus; sex differences

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INTRODUCTION

The developing brain is primed to respond to steroids by heightened receptor expression, elevated synthetic enzyme levels, and circulating binding globulins that sequester and possibly deliver hormones to select sights. Virtually all of the parameters that constitute brain development, proliferation, differentiation, migration, myelination, synaptogenesis, and apoptosis, are impacted or directly regulated by steroids (Fig. 1). Moreover, early hormone effects endure across the life span both in establishing the neuroarchitecture and in epigenetic imprinting. Yet, despite 50 years of inquiry our understanding of how, when, and why steroids direct brain development remains rudimentary, a state of affairs partly due to the complexity and multiplicity of effects of steroid hormones and partly due to technical and experimental limitations. This review seeks to briefly summarize current knowledge with an emphasis on animal models and the limited information available in humans and how that might relate to individuals with XXY/Klinefelter syndrome (KS).

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The Developing Brain is Permanently Altered by Exposure to Gonadal Steroids

Just as the gonadal anlage is considered bipotential for its capacity to differentiate

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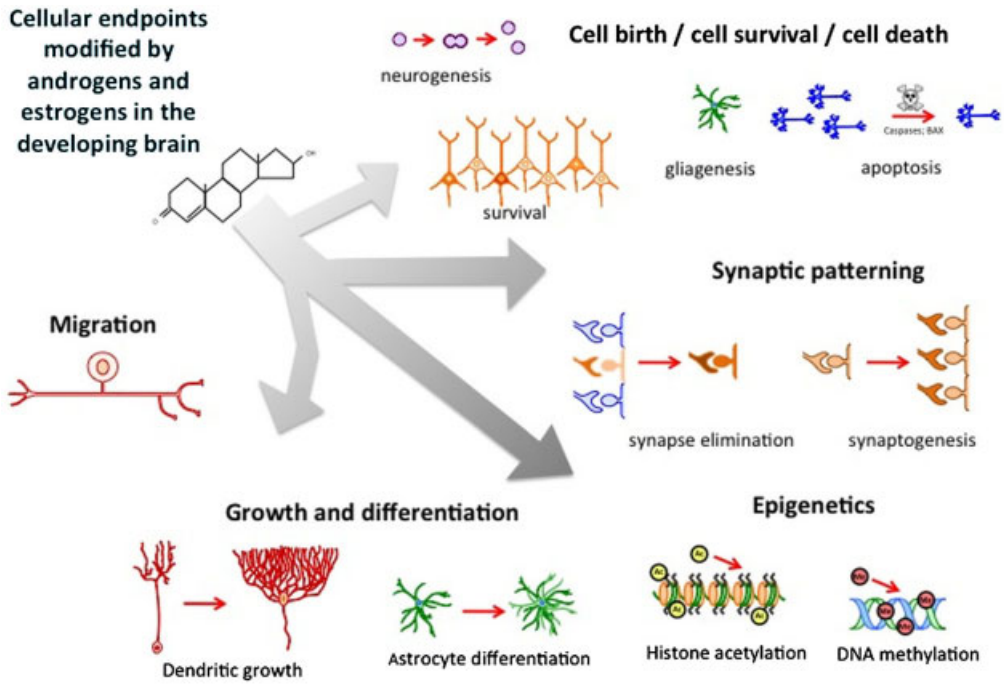

Figure 1. Steroids impact many endpoints in the developing brain. Multiple processes occur in tandem and in sequence in the developing brain and vary markedly by brain region and across time. Study of animal models reveals that steroid hormones exert powerful influences on virtually every parameter associated with brain development. Neurogenesis in the developing hippocampus is stimulated by androgens and estrogens and therefore occurs at a higher rate in the neonatal male compared to the female. Glial genesis in the amygdala is higher in females than males, whereas apoptosis is higher in some subregions of the female preoptic area but higher in males in other subregions. Together theses differences in cell birth and cell death act to sculpt the neuronal and glial population of particular brain regions. New neurons are also added to brain regions by migration and steroids can impact the speed and direction of migration. Once established, neurons grow, differentiate and make synaptic connections. Steroids can both promote or inhibit the establishment of synapses as well as alter the degree of dendritic growth and branching. Many of the neural architecture changes induced by steroids during the perinatal sensitive period are maintained throughout life via epigenetic changes to the genome, the precise mechanisms of which are still being elucidated.

into either an ovary or a testis, the brain is also bipotential in that it is equally capable of taking on a male versus a female phenotype. The determining variable is the hormonal milieu that the brain is exposed to during a critical developmental window and the determining variable of the hormonal milieu is the gonads and the attendant steroidogenesis. Studies in animals elucidated the principle of early hormonal impacts on the developing brain, now codified as the Organizational/Activational Hypothesis (Fig. 2). This concept was first articulated in a now iconic paper published in 1959 by Phoenix et al. [1959]. Based on experimental observations of guinea pigs, the Organizational/Activational Hypothesis simply states that adult responsiveness to gonadal steroids (the activational component) is dependent upon early hormonal exposure (the organizational component). In this initial study the endpoint of interest was sexual behavior. Females exposed to androgens in utero and resupplied with androgen in adulthood exhibited robust male sexual behavior towards other females (i.e., mounting and attempted intromitting), whereas females who were not exposed to androgens in utero but were given androgens as adults did not behave as males. Conversely, developing males deprived of their own androgens in utero (via treatment with inhibitors of steroidogenic enzymes or receptor antagonists) were immune to the activational effects of androgens in adulthood and failed to display any aspects of male sexual behavior. At the time that the Organizational/Activational Hypothesis was proposed it replaced earlier notions that sex specific behaviors were a product of peripheral organs or characteristics, so that a beagle with a penis would mount females simply because he had a penis, or a rooster would crow because he had a comb and wattle. While it was recognized that these peripheral characteristics were the products of hormones, it was not appreciated that the brain was a principle target of hormone action and that its developmental trajectory was permanently influenced by these potent molecules.

The sturdy framework of the Organizational/Activational Hypothesis has since stood the test of time and trial, having been extended to a range of physiological and behavioral endpoints and across numerous species, ranging from reptiles and amphibians to birds and most mammals [Morris et al., 2004; McCarthy and Arnold, 2011]. The dominant experimental animals of choice have been the laboratory rat and more recently the mouse due to the large litter size and in the case of

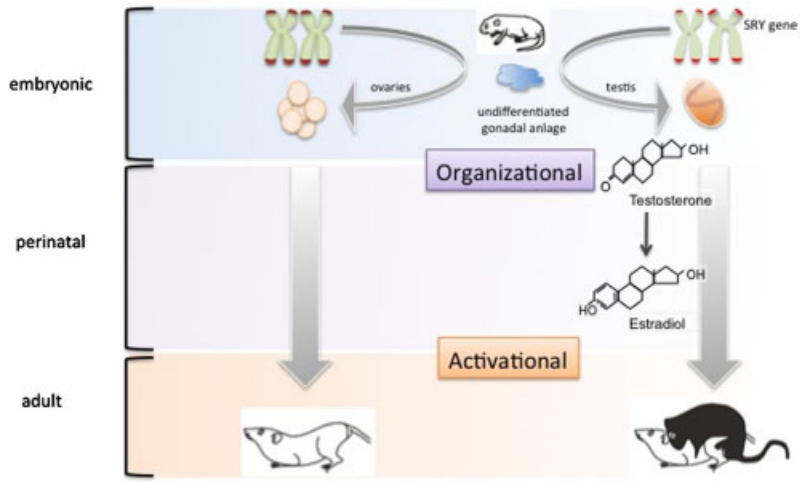

Figure 2. Organizational/activational hypothesis of steroid hormone effects on the brain across the life span. Chromosome compliment and the presence or absence of the Sry gene is the principle determinant of gonadal differentiation into either testes or ovaries during early embryonic development. During the perinatal period the male testis becomes active in steroidogenesis and produce copious quantities of androgens which gain access to the brain, and in rodents are largely converted to estrogens via the enzymatic process of aromatization. Combined androgens and estrogens then act on the brain to organize the neural substrate in a manner that will support adult male physiology and behavior when activated in response to postpubertal hormone production that is unique for males versus females. In this way a coordinated response between the gonadal phenotype and behavioral phenotype essential for successful reproduction is achieved.

the mouse, the power of genetic manipulation. An additional benefit of both species is the extension of the sensitive period into postnatal life, thereby allowing for the manipulation of individual pups without interfering with the dam. The ease and rapidity of work in rodents has elucidated many fundamental principles and novel cellular and molecular mechanisms. Critical among those principles is confirmation that the process of sexual differentiation occurs independently in different brain regions via distinct signally pathways initiated by the same steroid (i.e., androgen or estrogen). This produces a mosaic brain of varying degrees of maleness or femaleness in each subregion, greatly increasing individual variability in physiology and behavior [McCarthy, 2008, 2010]. The transference of this and other fundamental principles to non-human primates and humans has been achieved to varying degrees, but whether the same cellular mechanisms are at play in the primate brain has not yet been established.

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Differentiation of the Male and Female Brain Involves Three Distinct Processes

When considering sexual behavior or reproductive physiology as the endpoint, there are three distinct developmental processes recognized: (1) masculinization, (2) defeminization, and (3) feminization. Masculinization and feminization are the intuitively obvious processes whereby the brain is organized for the expression of sex-typic mating behavior and appropriate hormonal control of gonadotropin secretion from the anterior pituitary. That is, adult male rodents seek out and attempt to mount sexually receptive females, and those females in turn adapt a receptive posture referred to as lordosis. Regarding gonadotropin secretion, in females the cyclic hormone production from the mature ovary results in a surge of luteinizing hormone (LH) release from the pituitary which induces ovulation in the hormonally primed ovary. In males, LH production is pulsatile and relatively steady, as are testosterone levels. The female pattern of both sexual behavior and LH secretion are the default and the end product of the poorly understood process of feminization. Masculinization is the active induction of a neural network that promotes expression of male sexual behavior and a pulsatile steady release of LH.

The process of defeminization is most appropriately used in the context of sexual behavior in that it refers to a separate developmental process in which the neural network controlling female sexual responding, that is, lordosis in our animal models, is "removed" or "inhibited" so that males do not behave as females regardless of their hormonal profile. Masculinization and defeminization are partner processes in that they are both induced by the surge of androgen production during the perinatal period [Kudwa et al., 2005; Todd et al., 2005; Schwarz and McCarthy, 2008]. The evidence that there are two distinct processes, masculinization and defeminization, comes from the generation of adult animals that will display either male or female reproductive behavior or no sexual behavior when under the appropriate activational hormonal milieu (Fig. 3). Generating these animals requires either inducing masculinization in females without simultaneously inducing defeminization, meaning they retain their normal feminized phenotype, or conversely, disrupting masculinization in males without altering

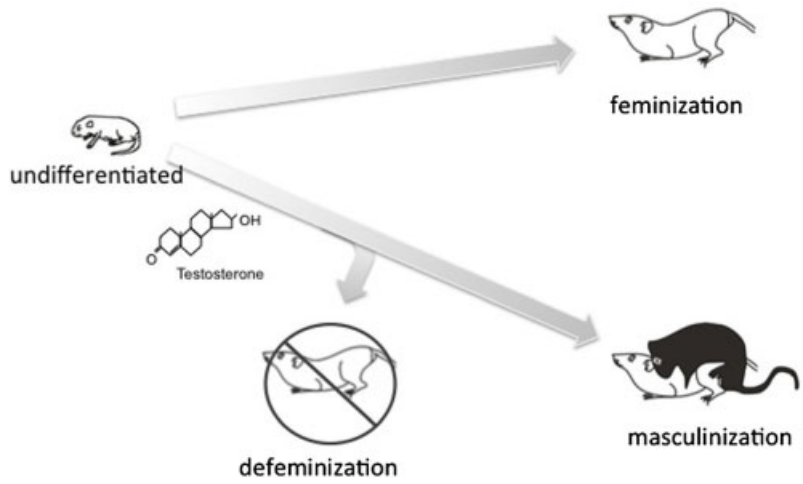

Figure 3. Masculinization, feminization, and defeminization. Study of animal models reveals three distinct processes that are organized by gonadal steroids during a developmental sensitive period. Feminization is the default pathway for brain development and will occur in the absence of any exposure to gonadal steroids. This active but poorly understood process organizes the brain to support the expression of female sexual receptivity in adulthood following activation by the proper sequence of hormonal exposure. Masculinization is the process whereby the undifferentiated brain is organized to support the expression of male sexual behavior in adulthood when testosterone levels are sufficiently high. Defeminization is the active removal of the organized substrate for feminized reproductive behavior. Empirical evidence proves this process is distinct from masculinization as opposed to a by-product, as it is controlled by different cellular mechanisms and neuronal populations.

defeminization. These individuals are asexual as adults, displaying neither male nor female behavioral patterns. Whether the term defeminization can be applied to other aspects of sexual differentiation of the brain is a matter of debate since by definition it requires there be two distinct morphs that can coexist. The majority of sex differences in brain and behavior do not involve true sexual dimorphism but instead are a continuum along which males and females may systematically differ to varying degrees [McCarthy et al., 2012].

The Cellular Mechanisms Mediating Masculinization, Feminization, and Defeminization are Regionally Distinct and Temporally Constrained

The existence of three distinct process of sexual differentiation of the brain was established over 30 years ago but impossible to prove as truly distinct until the cellular processes mediating the hormonal effects was elucidated. The surprising discovery that the downstream mediator of hormonally mediated masculinization of male sex behavior in rodents is a prostaglandin allowed for the treatment of females with a prostaglandin, and not a steroid, to thereby induce masculinization but not defeminization [Todd et al., 2005]. A series of subsequent studies determined that the major brain region mediated masculinization is the preoptic area, whereas the major sight of defeminization is the mediobasal hypothalamus and the ventromedial nucleus. Both are part of a wider neural network which includes reciprocal connections between them, but these serve as key nodal points. The brain region(s) mediating feminization of reproductive behavior have not been clearly identified but almost assuredly involve the same regions as those for male sexual behavior to some degree. What has been established is that the active feminization process occurs later in development than masculinization, involving a delayed hormonal surge, presumably from the ovary, around the second postnatal week [Bakker et al., 2003]. This discovery raises the specter of multiple sensitive periods with varying time courses and in distinct brain regions.

A series of subsequent studies determined that the major brain region mediated masculinization is the preoptic area, whereas the major sight of defeminization is the mediobasal hypothalamus and the ventromedial nucleus.

THE ORGANIZATIONAL/ ACTIVATIONAL HYPOTHESIS OUTSIDE OF REPRODUCTION

Progressing directly from studies on the hormonal organization of adult reproductive behavior and physiology was exploration of non-reproductive endpoints that exhibited sex differences and that may or may not have developmental origins. The hippocampus became an intense focus of attention with the startling report that estradiol increased dendritic spine synapse density by 30% when given to hormonally deprived adult females [Gould et al., 1990; Woolley and McEwen, 1992]. No previous physiological variable had been found to have such a robust effect on synaptic patterning and initial reports were met with a high degree of skepticism. Several decades later the hormonal modulation of the hippocampus remains a dynamic and intense area of investigation, revealing multiple novel mechanisms of synaptogenesis, and elucidating general principles that have subsequently been applied to other brain regions [Woolley, 2007].

The hippocampus is a brain region of interest for two reasons. One is its central role in feedback inhibition of the stress response axes and evidence that chronic or poorly controlled stress responding can both damage the hippocampus and be an underlying cause in

the development of mood disorders [Knable et al., 2004; McEwen, 2007]. The second is the essential role of the hippocampus in learning and memory. Both short-term and long-term memory consolidation require an intact hippocampus, particularly in regards to spatial learning [Fortin et al., 2002; Epp et al., 2007]. Males and females of many species show different profiles of stress responding as well as performance on spatial learning tasks. It has been presumed that sex differences in the hippocampus underlie the divergent responses of males versus females in stress and learning paradigms. The world wide female bias in depressive disorders has further focused attention on the hippocampus. Directly associating sex differences in the hippocampus to sex differences in physiological or psychological endpoints is difficult, as it is with any brain region, but one illustrative case is that of eye-blink conditional learning. In this task a rodent learns to blink its eyes in advance of an aversive shock or puff of air that it has associated with a conditioned stimulus such as a tone. Male and female rats perform equally well at learning this task. However, if rats are stressed in advance of the learning task, males will perform better than unstressed males and show an associated increase in hippocampal pyramidal neuron dendritic spine synapses while stressed females do the opposite, they perform worse and have a decrease in dendritic spine synapses [Shors et al., 2001; Shors, 2006]. This is an example of a sex divergence, an instance in which the sexes are the same under basal conditions but diverge in response to a challenge. Whether the deterioration in performance of stressed females is due to a heightened stress response compared to males is unknown, as stress responding is a complex, situational and experience dependent phenomenon. What is known, however, is that the hormonal modulation of dendritic spine synapses in the hippocampus is markedly different in males versus females. As noted before, the treatment of hormonally deprived females with physiological levels of estradiol, that is, hormone replacement therapy, increases dendritic spines synapses by 30%. Depriving males of hormones via castration also causes a dramatic loss of hippocampal synapses but despite high levels of aromatase and estradiol in male brains, only androgen is capable of restoring the synaptic profile to that of the gonadally intact male. Interestingly, females will respond equally well to estrogens or androgens as synaptogenic agents [Leranth et al., 2004; MacLusky et al., 2006; Hajszan et al., 2008]. The origins of the sex difference in sensitivity may be developmental as there is a divergence in signaling pathways associated with activated estrogen receptor in the hippocampus and this is determined developmentally [Meitzen et al., 2012]. At the cellular level the mechanistic basis for this organizational sex difference in adult hippocampal synaptic plasticity has not been established but is an active topic of investigation.

A third related reason for the disproportionate interest in the hippocampus is the discovery of active adult neurogenesis in the dentate gyrus, a component of the hippocampal complex [Cameron and McKay, 1998]. Only two brain regions in rodents and non-human primates retain the ability to generate new neurons throughout life, the subventricular zone (SVZ) and the subgranular proliferative zone of the dentate gyrus in the hippocampal formation. In humans, only the dentate gyrus appears capable of continuing to generate new neurons in adulthood [Sanai et al., 2011]. Both stress responding and vulnerability to mood disorders and learning and memory have been functionally linked with adult neurogenesis [Hanson et al., 2011; Eisch and Petrik, 2012; Marin-Burgin and Schinder, 2012; Shors et al., 2012; Yao et al., 2012], prompting inquiry into potential sex differences in this process. Adult female rats show higher rates of cell proliferation in the dentate gyrus, and this is largely due to the effects of estradiol [Tanapat et al., 1998; McClure et al., 2012]. However many variables impact on adult neurogenesis, ranging from stress to sexual experience to diet to social defeat [Glasper et al., 2012]. Thus hormones are only one variable, but certainly an important and confounding one given that many of the other important parameters impacting neurogenesis are also profoundly influenced by steroids.

In the neonate it is easier to eliminate the many confounding variables of adulthood, or at least to more carefully control for them, and an emerging consensus agrees that developmental events are antecedents to adult health and disease [Bale et al., 2010]. Brain areas critical to cognitive and other higher order functions tend to develop later than those involved in homeostatic control. This includes the hippocampus and dentate gyrus in which neurogenesis continues well into the postnatal period.

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In contrast to the adult where females have a higher rate of new cell proliferation, neonatal male rats generate almost twice as many new neurons per unit time during the first few days of life as females (Fig. 4). The male bias in neurogenesis is temporary, with both sexes dropping to a low baseline by the end of the first week of life. Moreover, clearly the male must experience compensatory cell death at some developmental time point as the adult hippocampus is not substantially larger in males compared to females. Nonetheless, the sex difference in neonatal neurogenesis is also hormonally dependent. Treatment of newborn females with a masculinizing dose of either testosterone or estradiol increases neurogenesis levels to that of males and antagonizing estrogen action in males

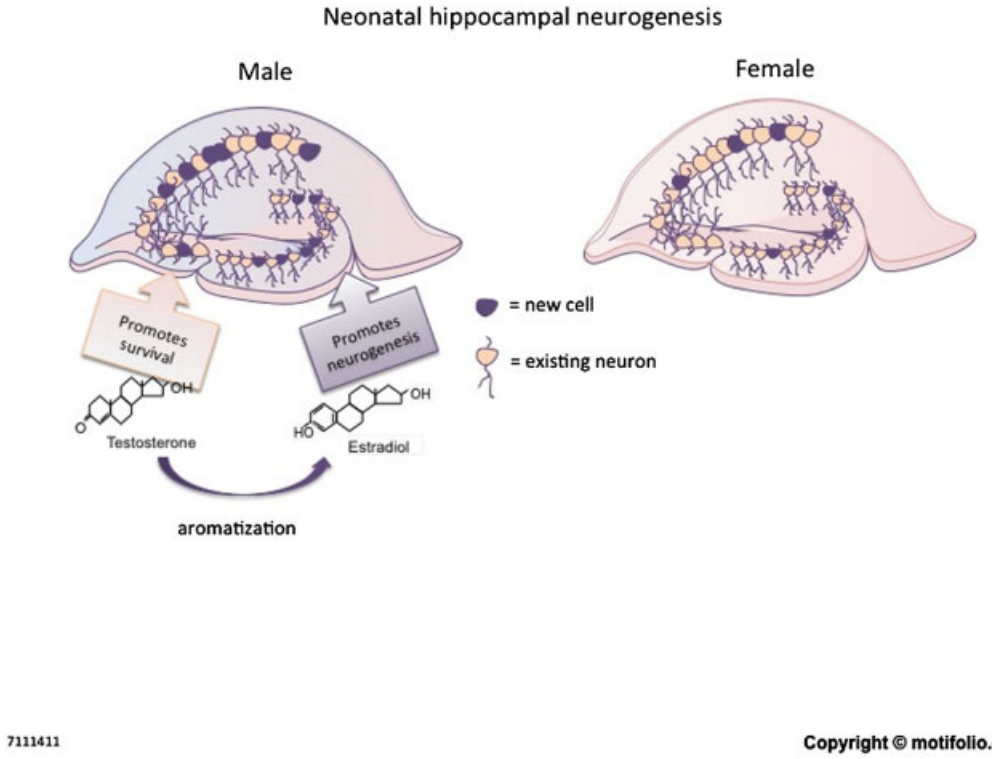

Figure 4. Sex differences and hormonal modulation of neurogenesis in the developing hippocampus. In the neonatal hippocampus more new cells are born and these are destined to become neurons. The sex difference is directed by the dual actions of androgens and estrogens, with the latter promoting neuronal precursor proliferation and the former enhancing the survival of the newly born cells until they can be fully integrated into the neuronal network. The hippocampus is a critical brain region in the control of spatial learning and in stress and anxiety responding. Both these parameters exhibit complex sex differences which are context and experience dependent and of which the origins remain poorly understood. Whether early sex differences in neurogenesis contribute to the divergence in hippocampal-dependent physiology and behavior in males and females remains to be determined.

has the opposite effect, reducing rates of cell proliferation to below even that of females [Zhang et al., 2008; Bowers et al., 2010]. This raises the interesting question of what is the function of double the rate of neurogenesis in newborn male rat pups, particularly since it is occurring during a developmental period characterized by little other than sleeping and nursing, although olfactory learning is certainly occurring. Intriguingly, there is a reappearance of the sex difference in neurogenesis in juvenile animals, several weeks after weaning but also several weeks from sexual maturity. The reemergence of the sex difference is the result of epigenetic imprinting during the early neonatal period mediated by estradiol [Bannerjee, McCarthy et al., unpublished observation], illustrating the power of hormones to exert enduring effects on brain and behavior. The interplay between steroids, brain development and epigenetics is discussed in further detail below.

The amygdala is another brain region involved in emotional and cognitive processes. Particular subdivisions are critical to aspects of learning, especially aversive learning or fear [Davis, 1997; Blanton et al., 2010], while others are essential to non-reproductive social behaviors such as play or individual recognition [Meaney et al., 1983; Cooke et al., 2000; Cooke and Woolley, 2005]. The amygdala is also notable for its sensitivity to androgens which persist throughout life [Romeo and Sisk, 2001], with unusual plasticity being maintained in the adult so that androgen deprivation reduces the volume of the amygdala while hormone replacement restores it [Cooke et al., 1999]. This has important implications for individuals diagnosed with XXY (KS) well into adulthood.

The juvenile period of the majority of mammalian species is characterized by a high level of social interaction, sometimes referred to as "rough and tumble play." This name comes largely from the fact that male juveniles, be they puppies, lions, colts, rats, rabbits, or boys, exhibit a higher frequency and greater intensity of physical interaction with other males than do females with other females. One of the aspects that makes this sex difference so interesting is that it occurs in the absence of any circulating gonadal steroid levels as it is during the quiescent period between the perinatal hormone surge and puberty. Thus the degree of play is taken as an indicator of prior hormonal influence. Androgens acting in the amygdala during the perinatal

sensitive period is a prerequisite for the intense play shown by juvenile males, and treating newly born females with androgens will increase the amount of rough and tumble play they engage in as juveniles [Auger and Olesen, 2009]. Interestingly, treating newborn female rats with an endocannabinoid agonist also increases the level of play they exhibit as juveniles, but has no effect on their male littermates [Krebs-Kraft et al., 2010]. Endocannabinoids are membrane derived signaling molecules that rapidly and locally modulate synaptic transmission. The name derives from the ability of cannabis to activate endocannabinoid receptors, thus endocannabinoids being referred to as the brains own marijuana. Assessment of endocannabinoid tone in the amygdala of development male and female rats reveals that males have a higher overall tone, meaning more endocannabinoid available at any given time. The higher endocannabinoid tone in males is correlated with decreased proliferation of astrocytes, specialized glia that play an essential role in synaptogenesis, as well as other vital functions. How a sex difference in the number of astrocytes in the amygdala might be determinant in the level of rough and tumble play exhibited as a juvenile is among the many unknowns of neural control of complex behaviors.

THE BRAIN AS A GONAD—LOCAL STEROIDOGENESIS IN DISCRETE BRAIN REGIONS

The production of copious quantities of steroids by the gonads is a key step to integrating the entire body for purposes of reproduction. The differentiation of the reproductive track, development of secondary sex characteristics, positive and negative feedback control of gonadotropin secretion from the anterior pituitary, and direct stimulation of gamete production in the case of males are all essential functions of gonadal steroids. The motivation to seek out mates and the physical acts of copulation are also behaviors controlled by steroids and the expression of these behaviors is directly correlated with the level of steroid detected in the circulation. Because the levels of steroid in the blood stream are so high and easily accessed and measured, there was little reason to consider that steroids might originate from anywhere other than the gonads and adrenals. The ability of the brain to aromatize androgens into estrogens was well known, but the sole source of the androgens was considered to be the gonad. However surprising observations of estrogen production by neurons cultured in a dish, and thereby deprived of any gonadally derived steroids, began to change this view [Rune et al., 2002; Prange-Kiel et al., 2003]. We now know that, at the least, the hippocampus and the cerebellum actively make estrogens de novo from cholesterol, possessing all of the requisite enzymes and thereby also synthesizing detectable quantities of androgens and progestins [Hojo et al., 2004; Dean and McCarthy, 2008; Dean et al., 2012b]. The 5-alpha reductase enzyme is found in abundance throughout the brain, leading to local synthesis of DHT as well [Melcangi et al., 1988]. A broad survey of the developing rodent brain reveals marked differences in the amount of testosterone, DHT and estradiol between brain regions [Konkle and McCarthy, 2007]. As would be expected based on the relative amount of aromatase, there is 10-fold higher levels of estradiol in the reproductively relevant POA and hypothalamus compared to the hippocampus and cortex, yet the estradiol found in these regions is highly potent and exhibits clear patterns unrelated to circulating steroid levels. There is a delayed (from birth) peak in estradiol production in the cortex and the timing differs by several days in males versus females. There are also late peaks in androgen production in the cortex, and more interestingly, the amount of androgen detected in the hippocampus and cortex is equal to or higher than that of the reproductively relevant regions (Fig. 5). The function of locally synthesized estradiol in the developing hippocampus is beginning to be elucidated, as noted above in the discussion of neonatal neurogenesis, but the role of androgens on the development of these brain regions remains an active but understudied topic of investigation. In the adult, locally synthesized estradiol promotes learning [Vierk et al., 2012] and acts presynaptically to regulate excitatory neurotransmission [Huang and Woolley, 2012]. The variables that regulate local steroidogenesis are not well understood although not surprisingly there is an impact of neural excitation and the subsequent calcium influx [Balthazart and Ball, 1998]. But by and large this also remains an under explored area.

The cerebellum is often studied in isolation from telencephalic and diencephalic brain regions, although emerging interest in its potential role in autism is beginning to change that. The principle neurons of the cerebellum, the Purkinje cells, express high levels of androgen receptor [Dean and McCarthy, 2008], but the research emphasis to-date has been on estrogens. Locally synthesized estradiol regulates dendritic spine synaptogenesis [Abel et al., 2011] as well as arborization of the dendritic tree [Dean et al., 2012b] during the first few weeks of life. Here again there is a surprising role for prostaglandins in that PGE2 stimulates aromatase activity within Purkinje cells and increases locally produced estrogens. There is a basal level of estradiol production regulated by PGE2 but this is also increased by inflammation following LPS injection to neonatal rat pups. If inflammation occurs during a restricted sensitive period of Purkinje neuron development, there is an enduring change in social play during the juvenile period, but in this instance only in males who exhibit reduced social play and increased perseveration in attention to inanimate objects [Dean et al., 2012a], characteristics consistent with autism spectrum disorder in humans.

SEXUAL DIFFERENTIATION OF THE PRIMATE BRAIN

When possible the fundamental principles of the Organizational/Activational Hypothesis have been applied to primates, specifically the Rhesus Macaque [Wallen and Baum, 2002; Wallen, 2005]. In general the principle holds with two important variants. First is that

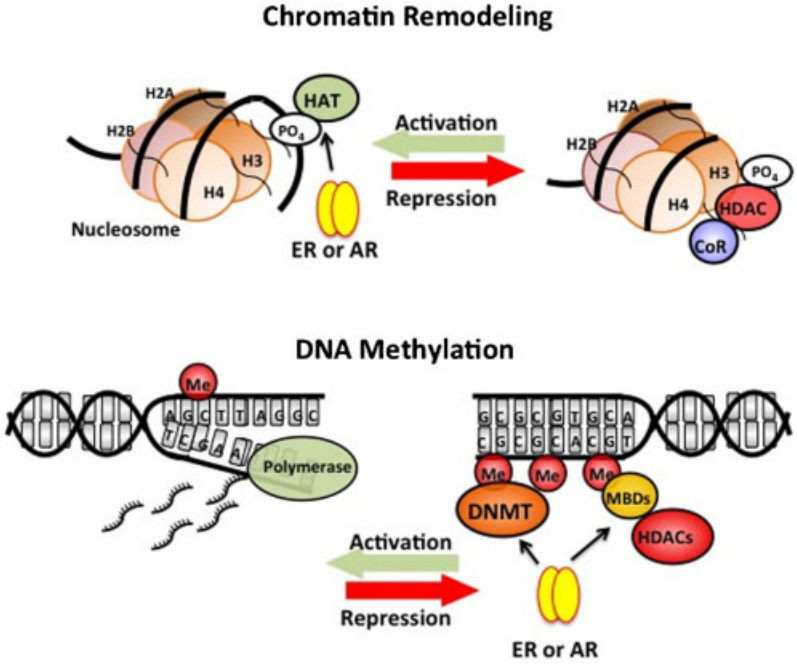

Figure 5. Hormones direct epigenetic changes in the brain. The two dominant forms of epigenetic modification are changes to the chromatin via modifications of the histones (H2A, 2B, 3, and 4) and direct methylation (Me) of cytosine residues in the DNA. Steroid receptors such as androgen receptor (AR) and estrogen receptor (ER) form complexes with other proteins that possess enzymatic activity that can directly modify the histones, such as HAT (histone acetyltransferase) which leads to activated gene expression, and indirectly by altering the activity of the DNMTs (DNA methyl transferase) and MBDs (methyl binding domain) proteins. Together these epigenetic modifications can induce enduring changes that may be a component of the underpinnings of the organizational effects of hormones on the developing brain.

masculinization of brain and behavior in Macaques occurs entirely prenatally, with no clearly identified role for postnatal hormones, although with the caveat that the number of endpoints assessed is limited by the cost and ethical constraints of primate research. The second important difference is the capacity of estrogens to masculinize the brain, which is dominant in rodents and either absent or greatly reduced in primates. Instead, androgens, presumably directly activating androgen receptors, are the dominant masculinizing hormones in primates. Some argue that this renders the work in rodents meaningless as the primary hormone mediating the process is different. But this belies the fact that hormones are only the initiators of a signaling cascade and it is the subsequent cellular processes that functionally differentiate the nervous system. Androgens and estrogens often converge on similar signaling pathways, and thus only by understanding the mechanisms of hormone action, at the cellular and molecular level, can we ask whether the process of brain sexual differentiation is indeed different in primates. The elucidation of mechanism is a process best achieved in rodents. Again, in view of the ethical constraints of research on primates, translation of mechanism will likely best be achieved by indirect or inferential means, further increasing the importance of precise determination of signaling pathways in rodents.

Is the Human Brain Sexually Differentiated?

This is a far more challenging question than it first appears. If we assume that as just another species humans must be subject to the same rules and regulations as in others, then the answer would seem to be an unqualified yes, of course the human brain is sexually differentiated. But when we consider the enormous impact of environment and experience on the human brain, which in our particular case means cultural and societal expectations that are unique to humans, then we have to include these variables in our analyses and recognize their impact. The truth probably lies somewhere in the middle, there is a biological as well as a cultural impact on brain and behavior in boys and girls, men and women. Parsing out the relative contribution of each is difficult as we cannot conduct controlled experiments and so must rely on indirect evidence, but progress has been made via so-called "natural experiments." These are instances in which the hormonal profile of individuals is disrupted or altered in some way due to genetic mutations or exogenous treatments intended for other purposes (Table I). An example of the latter is the now defunct practice of treating pregnant women with the potent estrogen diethyl stilbestrol, or DES, in order to maintain a healthy pregnancy. In addition to the focus on increased risk of clear cell adenocarcinoma of the cervix and vagina in females, emphasis here has been largely on reproductive track abnormalities in males. Some hints of psychosexual and gender identity issues have emerged from various patient interest groups, and there are reports of small but significant increases in bisexuality and homosexuality in DES exposed women [Ehrhardt et al., 1985; Ehrhardt et al., 1989] but for the most part the effects are small and inconsistent and therefore not sufficient to warrant substantial investigations [Kebir and Krebs, 2012]. That there are no profound effects of DES exposure on psychosexual development is consistent with the view that androgens, not estrogens, are the dominant masculinizing hormone in primates. There are several examples of naturally occurring mutations that result in altered androgen exposure, the best characterized being: (1) androgen insensitivity, (2) 5-alpha reductase deficiency, and (3) CAH or congenital adrenal hyperplasia. Androgen insensitivity is the complete or partial loss of response to endogenous androgens due to spontaneous mutations in

TABLE I. Conditions in Which Variations in Hormone Exposure or Sensitivity Allow for Assessment of the Biological Contribution to Sexual Differentiation of the Human Brain

Condition	Hormonal impact	Effects on sexual differentiation of the brain
DES exposure in utero	Elevated estradiol during brain development	Inconsistent or modest effects on masculinization or feminization
Estrogen insensitivity	Exceedingly rare but recently discovered men with mutations in the aromatase gene or estrogen receptor	Mild reduction in libido but otherwise normal
Androgen insensitivity syndrome	Spontaneous mutation of the androgen receptor gene results in complete or partial loss of cellular sensitivity to androgens	XY males develop as behaviorally phenotypic females
5-Alpha reductase deficiency	Reduced production of DHT leads to incomplete masculinization of genitalia at birth, masculinization proceeds at puberty	Majority of XY individuals choose to live as males post puberty
Congenital Adrenal hyperplasia (CAH)	Elevated androgen exposure in utero, girls born with partially masculinized genitalia	Small but significant increase in homosexuality, significant shift towards male-typic behaviors but within the normal range
Klinefelter syndrome (XXY)	Reduced androgen exposure in males throughout life	Behavioral changes and significant deficits in speech and language capabilities

the androgen receptor. When the loss of sensitivity is complete an XY individual develops as a phenotypic female and psychosexual differentiation is also completely female, which is also consistent with the notion that androgens are the masculinizing hormone in primates, including humans [Brown et al., 1990; French et al., 1990; Cohen-Bendahan et al., 2005]. Cases of 5-alpha reductase deficiency are rare but occur with some frequency in a small population in the Dominican Republic. In this instance individuals make testosterone but little to none of the highly potent metabolite dihydrotestosterone or DHT. As a result, XY males are under virilized and this can be sufficiently severe that they are mistaken for females [Bertelloni et al., 2007]. In the Dominican Republic population there is sufficient androgen production at puberty that sexual differentiation proceeds to the degree that most individuals become masculinized and gender assignment is considered to "switch" from female to male. This was initially viewed as evidence of the enormous plasticity of gender in humans and argued against the notion that the human brain was sexually differentiated in the same way as animals [Hines, 2002, 2004]. This conclusion has subsequently been essentially retracted as it has become apparent that individuals with 5-alpha reductase deficiency in this population are generally identified at birth and are considered more of a third phenotype as opposed to normal girls who then become normal boys. This, combined with more recent reports on gender assignment and reassignment in indivduals with 5-alpha reductase deficiency [Mendez et al., 1995] as well as some spectacularly failed attempts to gender reassign boys as girls based on penis size (either microphallus or loss due to damage), has led to the conclusion that any substantial progress down the path towards masculinization in a male should be completed rather than reversed. However, the response to gender reassignment is not always predictable, leading to a movement to delay such decisions as long as possible so that the child can be an active participant if possible [Meyer-Bahlburg, 2001].

The richest data set regarding human brain sexual differentiation comes from the study of CAH girls. The dominant form of CAH is the result of mutations in the 21-beta-hydroxylase enzyme, a critical enzyme in the production of glucocorticoids and mineralocorticoids. The blockade of this synthesis pathway leads to an excess of precursors in the androgen pathway and therefore over production of testosterone and DHT. In females this can result in significant virilization which is usually not detected until birth. The psychosexual differentiation of CAH girls has been the topic of intense study in populations in the US, Japan, and Europe [Iijima et al., 2001]. There is a small but significant increase in the percentage of CAH girls self-identifying as lesbian but the overwhelming majority of CAH girls identify as female regardless of sexual preference [Meyer-Bahlburg, 2001]. Of particular interest, however, are measures of psychological state. This has been addressed in several ways but the most compelling is the work conducted by Melissa Hines focusing on toy choice. Play behavior peaks between the ages of 3–6 and both the type of play and choice of playthings varies in systematic ways between boys and girls [Jadva et al., 2010]. With the use of one-way mirrors, Hines has been able to quantify the toy selections of boys, girls and CAH boys and girls when presented with an array of items to choose from. Among the array are toys that have previously been documented to be preferred by boys, and vice versa, those preferred by girls. There is also a selection of toys deemed gender neutral, such as puzzles or picture books. Testosterone measured in infancy

positively correlates with bias towards preference for boy toys in unaffected boys and girls [Lamminmaki et al., 2012]. The toy choice of CAH boys is not different from unaffected boys, but the choice of CAH girls is clearly skewed away from girl-preferred toys and biased towards boy-preferred toys, although there is also an influence of parental socialization on the toy choice of all the children [Wong et al., 2012]. While not without caveats, these observations speak to the profound impact of prenatal hormone exposure on the human brain in a realm that is outside of reproduction per se. What is particularly notable about this approach is that children of this age do not have circulating gonadal steroids and thus any observed sex differences are a reflection of past exposure, not current.

XXY (KS) in the Context of Sexual Differentiation of the Brain

XXY (KS) defines individuals with a sex chromosomal polyploidy that involves one Y and two or more X chromosomes. Most males with XXY (KS) are infertile and have reduced testicle size and an associated reduction in androgen production which in turn generates a myriad of phenotypic alterations although largely still within the normal range [Groth et al., 2012]. There has been little to no consideration of the role of sexual differentiation of the brain in individuals with XXY (KS) nor have the implications of both the syndrome itself and the impact of hormone replacement therapy to patients been considered in the context of evidence for sexual differentiation of the human brain. This may in large part be due to the enormous variability in clinical presentation of the syndrome, as well as late and under diagnoses of the condition. Measures of the psychosexual and cognitive capacities of adolescent boys with XXY (KS) reveals striking analogies to girls with CAH in that while there are significant differences in cognitive and psychosexual parameters, they remain within the normal range and most males identify as heterosexuals, although interest in girls is reduced compared to that of XY boys. On intelligence tests, the dominant variable reduced in boys with XXY (KS) was verbal IQ, which may contribute to some of the social discomfort often experienced by these boys. A recent comprehensive review of clinical findings confirmed that the dominant cognitive impairment is in the area of language processing and notes that many boys with XXY (KS) require and benefit from speech therapy [Groth et al., 2012].

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Reports of cognitive sex differences in humans that generate the greatest amount of interest, both positive and negative, are those involving verbal abilities and mathematical abilities. It is not possible to model mathematical abilities in animals, and the interested reviewer is referred to an excellent review on the topic of human sex differences in cognitive abilities [Spelke, 2005]. Until recently there was also little opportunity to model verbal abilities, other than in birds which are considered the only species besides humans that actively learns and adapts their vocalizations. This situation was changed by the discovery of a specific gene, *FoxP2*, which is closely linked to verbal abilities in humans [Graham and Fisher, 2012] and has produced an explosion of interest in the biological basis of language acquisition. The discovery of the gene came from the geneology of an extended family with devastating language and speech impairments [Lai et al., 2001]. As a transcription factor, Foxp2 regulates both the repression and expression of a large number of genes, many of which are associated broadly with brain patterning and specifically with neurite outgrowth, dendritic branching, and axonal morphology [Fisher and Scharff, 2009; Schulz et al., 2010; Mukamel et al., 2011; Vernes et al., 2011]. The *Foxp2* gene is highly conserved across species and has been associated with song learning in birds and ultrasonic vocalizations in rodents. Generation of transgenic mice bearing the mutated form of the human *FOXP2* gene has further advanced understanding of this critical gene. We recently found a sex difference in both the mRNA and protein product of the *FoxP2* gene in the rodent in multiple brain regions. By manipulating gene expression we established a causal relationship between the amount of gene product and sex differences in ultrasonic vocalizations by neonatal rat pups. Interestingly, analyses of FOXP2 mRNA and protein in samples of human cortex in Brodmann's area 44, a language associated brain region, also revealed a sex difference in 4–5-year-old boys and girls [Bowers et al., 2012]. Recent studies of a rare variant of KS (49,XXXXY) revealed a significant positive effect of androgen treatment of infants and children on the speech and language domain, gestural communication, and vocabulary development [Samango-Sprouse et al., 2011, 2012]. A major implication of this work is the importance of early androgen treatment for enhancing cognitive development, particularly related to speech and language. Studies in our animal models indicate the sex difference in *FoxP2* is mediated by androgens [Bowers et al., 2012], leading to the question of whether there is a connection between reduced verbal performance in boys with XXY (KS) and the *FOXP2* gene.

EPIGENETICS AND SEXUAL DIFFERENTIATION OF THE BRAIN

There are two defining features of brain sexual differentiation which are shared

with many other developmental effects: (1) the process is restricted to a narrow developmental window or sensitive period and (2) the effects endure into adulthood. All multicellular organisms consist of differentiated cells and the maintenance of this differentiation is a function of epigenetic silencing of large numbers of genes. In this way a kidney cell remains a kidney cell as opposed to becoming a liver cell over time. Moreover, if a liver cell divides, it should give rise to new liver cells and not a kidney cell. The suppression of most genes is achieved via epigenetic changes to both the DNA and the surrounding chromatin. Together these serve the purpose of preventing access of transcription factors to the DNA and thus there is no gene expression in that region. However there are frequently instances in which a gene needs to be temporarily turned on or off, and this also can involve more labile epigenetic modifications, particularly to the chromatin. The most frequent modifications are acetylation and methylation which alter the charge on key amino acids of the histones and thereby constrain or relax the chromatin and alter access of transcription factors [Crews, 2008; Nugent and McCarthy, 2011]. Steroid hormones bind to nuclear receptors which in turn associate with a large number of co-regulatory factors, many of which possess the enzymatic activity capable of modifying the histones that form the core of nucleosomes, thereby acetylating or deacetylating key amino acids. Changes to the chromatin are complimented by direct changes to the DNA which largely involve methylation of cytosines that are located in proximity to guanines, referred to as CpG's. The frequency of CpG's in the genome is lower than would be predicted by chance and when they are found they tend to be in clusters in the promoter regions of genes, leading to the term CpG islands. Methylation of CpG's is considered an effective and often permanent means for suppressing gene expression.

In recent years there has been a surge of interest in the epigenetics of the brain as a means to explain why adverse or traumatic events can have such enduring and devastating consequences. The emerging and somewhat surprising consensus is that the brain is particularly sensitive to epigenetic modification and that even essential processes such as learning and memory involve changes to the chromatin and even the DNA [Champagne, 2008, 2012].

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This, combined with the already established relationship between steroids and histone modifications, made the question of epigenetics in sexual differentiation of the brain an obvious one to ask [McCarthy et al., 2009]. There have been two general approaches. The first is a descriptive approach, quantifying epigenetic modifications in specific brain regions of males and females and then determining if treatment of females with male hormones during the perinatal period shifts the epigenetic profile to that of males (Fig. 5). This has been found to be the case for both chromatin modifications and methylation of DNA, and it has also been found in both reproductively relevant brain regions like the hypothalamus and those more associated with cognitive and emotional responses such as the cortex and hippocampus [Tsai et al., 2009; Schwarz et al., 2010]. The second approach is to use pharmacological manipulations to inhibit or stimulate epigenetic modifications and determine the impact on sexually differentiated brain and behavior. This also has proven informative and confirmed that indeed steroid action early in life is permanently altering the epigenome in the male brain so as to maintain a masculinized phenotype through to adulthood and beyond [Matsuda et al., 2011]. The nuances and complexities of the epigenetic regulation of sexual differentiation of the brain are still being worked out, and whether the sensitive period can be extended or even eliminated by changing epigenetic marks is an intriguing implication of these findings but one yet to be established.

CONCLUSIONS

Sex differences in brain and behavior is a topic that continues to garner great interest as well as considerable suspicion. The suspicion is well placed in that history tells us that women are more likely to be disadvantaged by any discussion of innate abilities. Moreover, we cannot over estimate the impact of external expectations of gender appropriate behavior, which begin at the moment of birth, on the development of the brain. But there is pervasive evidence for a biological underpinning to some components of human behavior that differ on a systematic yet highly variable manner in boys and girls, women and men. By understanding that biology we can place it in appropriate context and make informed decisions regarding either gender assignment, as in the case of children with ambiguous genitalia or genital injury, and in circumstances such as that presented by XXY (KS) in which there is a documented decrement in androgen production. The more we understand how, when, where, and why steroids impact on the developing brain and how that in turn endures to influence adult brain and behavior, the better we can design and administer effective hormone replacement.

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