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Review article

Cellular and molecular mechanisms of sexual differentiation in the mammalian nervous system

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ABSTRACT

Neuroscientists are likely to discover new sex differences in the coming years, spurred by the National Institutes of Health initiative to include both sexes in preclinical studies. This review summarizes the current state of knowledge of the cellular and molecular mechanisms underlying sex differences in the mammalian nervous system, based primarily on work in rodents. Cellular mechanisms examined include neurogenesis, migration, the differentiation of neurochemical and morphological cell phenotype, and cell death. At the molecular level we discuss evolving roles for epigenetics, sex chromosome complement, the immune system, and newly identified cell signaling pathways. We review recent findings on the role of the environment, as well as genome-wide studies with some surprising results, causing us to re-think often-used models of sexual differentiation. We end by pointing to future directions, including an increased awareness of the important contributions of tissues outside of the nervous system to sexual differentiation of the brain.

1. Introduction

The National Institutes of Health (NIH) in the U.S.A. recently announced that grant proposals submitted after January, 2016 must include sex or gender in study designs, or explain why not (NIH Notice Number: NOT-OD-15-102). When sex differences are looked for, they are often found, so we are likely to be on the cusp of learning about a host of new sex and gender differences in the brain. Unraveling the underlying mechanisms will become a priority, and this is an opportune time to examine what research to date has taught us about how neural sex differences develop and change cellular function.

The NIH initiative came in response to observations that a disproportionate number of pre-clinical studies have for many years used only male subjects or cultured cells of unknown sex (Beery and Zucker, 2011; Blanchard et al., 1995; Sechzer et al., 1994). Over 70% of the basic research articles published in key neuroscience journals in a recent year used only males or did not specify the sex of subjects (Beery and Zucker, 2011), despite overwhelming evidence that there are important differences between male and female brains. Although the most prominent sex differences are often seen for reproductive functions, differences in other realms, such as cognition, energy balance, and stress responsiveness, are also well established (Bangasser and Valentino, 2012; Imwalle et al., 2006; Kimura, 2002; Mauvais-Jarvis, 2015). Moreover, the effects of some manipulations don't just differ by sex, but may push the brain and behavior in opposite directions in males and females (e.g., Oomen et al., 2009; Shors et al., 2001; Veenema et al., 2013; Waddell et al., 2008).

Several previous reviews on sexual differentiation of the mammalian brain and behavior are available (e.g., Forger et al., 2015; McCarthy et al., 2009). Here, we focus on neural sex differences for which the cellular or molecular mechanisms underlying the difference are known. By necessity, most of the evidence we present comes from work on rats or mice, since rodent species have been most amenable to studies at the cell and molecular level; when possible, we indicate where results apply more broadly. We attempt to present enough of the older and background material for those who may be new to the field, and also

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emphasize the most recent findings or those that illustrate a novel mechanism.

1.1. Agents of sexual differentiation

Broadly speaking, sex differences can be attributed to three interacting factors: sex chromosomes, gonadal hormones, and the environment. Although some neural sex differences develop under the direct influence of genes on the sex chromosomes (see Section 3), in most cases, effects of the sex chromosomes appear to be indirect, and mediated by hormones produced by the gonads. Only males inherit a Y chromosome from the father, and the *sex-determining region of the Y* (*Sry*) gene, in cooperation with downstream genes, induces the initially "bipotential" embryonic gonads to develop into testes (Kashimada and Koopman, 2010; Koopman et al., 1991). The fetal testes begin secreting testosterone within days after they differentiate (approximately embryonic day (E) 14 in the mouse, E16 in the rat, and week 10 of gestation in humans) and a wealth of data indicates that this hormone, or a metabolite, is responsible for masculinization of the brain as well as the periphery (Forger et al., 2015; McCarthy, 2011).

Testosterone can act via the androgen receptor, or may be converted (aromatized) to an estrogen at target cells by the cytochrome P450 aromatase enzyme, and subsequently act via estrogen receptors (Naftolin, 1994; Roselli et al., 2009). Conversion of testosterone to estradiol is required for full masculinization of many brain features in rodents (but see Zuloaga et al., 2008), whereas androgens are thought to play the dominant role in sexual differentiation of the brain in primates (Thornton et al., 2009; Wallen, 2005).

Effects of gonadal steroid hormones have traditionally been characterized as either "organizational" or "activational" (Phoenix et al., 1959). Organizational, or *programming*, effects outlast the hormone exposure: e.g., when hormone exposure early in life induces an enduring, or permanent change. Activational, or *acute*, effects are defined as those requiring the continued presence of the hormone. In many cases, both programming and acute actions of steroids are required. In the classic experiments by Phoenix et al. (1959), for example, the exposure of female guinea pigs to testosterone *in utero* permanently decreased female sexual behavior and increased male sexual behavior in adulthood, but this could only be seen when sex-specific copulatory behaviors were "activated" by treatment with the appropriate steroids in adulthood. Over 50 years later, most research in the field has shifted to rats and mice, and substantial progress has been made in understanding neuroanatomical and neurochemical changes that correlate with the differentiation of behavior, as well as the cellular and molecular mechanisms that may underlie these effects.

1.2. New(er) approaches to old questions

One puzzle presented itself early on in studies of sexual differentiation of the rodent brain: since steroids in maternal circulation can reach the fetus, why aren't all fetuses masculinized by maternal estrogens? The proposed solution was that alpha fetoprotein (AFP), an estrogen-binding protein that is highly abundant in fetal plasma, sequesters peripheral estrogens and keeps them from reaching the brain (McEwen et al., 1975). Other evidence, however, suggested that AFP might instead be a carrier protein delivering estrogens to the brain (Toran-Allerand, 1984). This issue was recently reexamined by studying the brain and behavior of *Afp* knockout mice. For most traits, female *Afp* –/– mice are convincingly male-like (Fig. 1; Bakker et al., 2006; Gonzalez-Martinez et al., 2008), supporting the idea that AFP normally protects female fetuses from circulating estrogens. There are some interesting exceptions, however: odor preferences and vasopressin immunoreactivity in the brain remain female-typical in female *Afp* knockouts (Bakker et al., 2006, 2007), suggesting that exposure to estrogens during embryonic development is not sufficient to masculinize these traits in mice. Although AFP is present in the plasma of fetal primates, including humans, it does not avidly bind estrogens (Aussel and Masseyeff, 1983), so is unlikely to protect the fetal brain from estrogen exposure. Other steroid binding proteins may play that role, or no such protection may be needed, if sexual differentiation of the brain and behavior in primates is primarily mediated by androgens (Thornton et al., 2009; Wallen, 2005).

A second question concerns the relationship between levels of gonadal steroids in the blood and those in the brain. Testosterone secretion begins soon after testis differentiation, and a second surge occurs at birth (Resko, 1985; Reyes et al., 1974; Weisz and Ward, 1980). Brain levels of androgens and estrogens may not simply reflect plasma levels, however. A recent radioimmunoassay study found that neural levels of androgens and estrogens vary by age and brain region in complex patterns that do not correlate with peripheral hormone levels and cannot be explained simply by known levels of aromatase (Konkle and McCarthy, 2011). Even more surprising, combined gonadectomy and adrenalectomy of rats on the day of birth did not alter brain levels of estrogens or androgens three days later (Konkle and McCarthy, 2011). The answer to this puzzle may lie in the brain itself being capable of steroid synthesis *de novo* (Robel and Baulieu, 1995). Many new roles for neurosteroid production have been discovered in the last decade (Krentzel and Remage-Healey, 2015; Micevych and Sinchak, 2011), but mainstream theories of sexual differentiation of the brain have not yet incorporated this concept, which makes this an area ripe for investigation.

2. Cellular bases of sex differences

While prenatal testosterone exposure differentiates the periphery, the neonatal testosterone surge is most closely linked to sexual differentiation of the brain and behavior in rodents. In principle, gonadal steroids could cause sex differences by altering any of the major neurodevelopmental events: neurogenesis, migration, differentiation of phenotype, or cell death (reviewed in Forger, 2006, 2009). However, the majority of neurogenesis and cell migration is complete prior to birth (i.e., before the neonatal testosterone surge), which makes it less likely that hormones act on these processes to differentiate the brain.

An exception is that many hippocampal neurons are generated postnatally, and newborn male rats have a two-fold higher rate of cell birth in the hippocampus than do females (Zhang et al., 2008). The rate of hippocampal cell genesis in females can be increased to male levels by neonatal treatment with either testosterone or estradiol and, in the CA1 region, many of the newborn cells differentiate into neurons (Bowers et al., 2010). Sex differences in the size of the hippocampus in adults are subtle, however, so the sex difference in the production of new cells may be offset by differences in cell loss at some point in development.

Evidence of a role for migration in sexual differentiation of the brain comes from studies using live-cell fluorescent video microscopy to study cell movements in slice cultures of the embryonic brain (Henderson et al., 1999; Knoll et al., 2007). For example, neurons in the preoptic area/anterior hypothalamus (POA/AH) of E14 female mice move faster and more frequently than those in males (Knoll et al., 2007). Administration of estradiol decreases the rate of movement of dorsal POA/AH cells while increasing the movement of cells located more ventrally (Knoll et al., 2007). There is also a sex difference in the location of neurons expressing estrogen receptor β (ERβ) in the anteroventral