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The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues

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Abstract

The 1959 publication of the paper by Phoenix et al. was a major turning point in the study of sexual differentiation of the brain. That study showed that sex differences in behavior, and by extension in the brain, were permanently sexually differentiated by testosterone, a testicular secretion, during an early critical period of development. The study placed the brain together in a class with other major sexually dimorphic tissues (external genitalia and genital tracts), and proposed an integrated hormonal theory of sexual differentiation for all of these non-gonadal tissues. Since 1959, the organizational-activational theory has been amended but survives as a central concept that explains many sex differences in phenotype, in diverse tissues and at all levels of analysis from the molecular to the behavioral. In the last two decades, however, sex differences have been found that are not explained by such gonadal hormonal effects, but rather because of the primary action of genes encoded on the sex chromosomes. To integrate the classic organizational and activational effects with the more recently discovered sex chromosome effects, we propose a unified theory of sexual differentiation that applies to all mammalian tissues.

Keywords

testosterone; estradiol; organizational; activational; sex chromosome; X chromosome; Y chromosome; sexual differentiation; sex difference

Ever since 1959

The 1959 publication of the paper by Charles H. Phoenix, Robert W. Goy, Arnold A. Gerall, and William C. Young is appropriately perceived as a major turning point in the study of sex differences in the brain. These authors provided a conceptual framework that has been repeatedly tested and improved since 1959, but has not been substantially undermined by experimental findings in the intervening half century. That's remarkable. The methods used by Phoenix et al. continue to be emulated today in any comprehensive study of sex differences in the brain and behavior, or in non-brain phenotypes (Becker et al., 2005). The framework has been expanded to explain a large majority of sex differences in phenotype of all non-gonadal tissues (e.g. Beatty, 1984; Greenspan et al., 2007). In addition, it has been applied progressively more broadly to new levels of analysis (cellular, molecular, genetic) of sex differences as they became possible in the last 50 years. Along the way, a few amendments were made to the framework, which have served to enhance it. We begin by discussing what Phoenix et al. found and what they concluded, and then discuss some of the

“footnotes” that have been added to the framework based on subsequent research. We then discuss sex differences that are not explained by the organizational-activational framework, and merge those findings with the organizational-activational concept to suggest a unified theory of sexual differentiation of all tissues in mammals.

The conceptual framework of Phoenix, Goy, Gerall, and Young: Organization and Activation

Phoenix et al. injected pregnant guinea pigs with testosterone propionate, and then studied the mating behavior of the offspring when they were adult. They were interested in the behavioral capacity of the animals, defined by whether the experimentally manipulated guinea pigs would behave like a male or female. If the animal showed lordosis behavior, they concluded that it had the capacity to show behavior typical of females. If the animal mounted a receptive female guinea pig, they concluded that it had the capacity to show behavior typical of males. It was important to test the animals under conditions that normally lead to high frequencies of the behaviors. Thus, to test for lordosis, the animals were gonadectomized before puberty and as adults injected with estradiol benzoate followed by progesterone, and then stimulated manually (“fingered”) in a manner that reliably elicits lordosis in control females. The hormones injected were thought to mimic the hormones that bring about the female guinea pig’s behavioral heat, and the manual stimulation mimicked the tactile stimuli normally provided by the copulating male. In contrast, to test for masculine mounting, the animals were gonadectomized before puberty and then injected with testosterone propionate as adults and exposed to a receptive female.

The major findings of the paper were as follows.

1. *Fetal masculinization.* Females treated prenatally with testosterone showed less lordosis and more mounting as adults than control females. The authors concluded that exposure to testosterone during fetal life makes female guinea pig behavior more like that of males. The implication (almost tacit in their 1959 article) was that the male is normally masculinized by testosterone secreted by his testes during fetal life. (Phoenix et al. did not use the term “defeminization” presumably because they viewed masculinization as involving both an increase in masculine behavior and decrease in feminine behavior; see below for further discussion).
2. *Permanence.* The prenatal effects were permanent, since they were observed months after the end of the fetal testosterone treatment. The activational effects of gonadal hormones were seen as acute and reversible, not permanent.
3. *Organizational.* The effects of prenatal testosterone were interpreted to have changed the response to gonadal hormones that activate behaviors in adulthood. “The data are uniform in demonstrating that an androgen administered prenatally has an organizing action on the tissues mediating mating behavior in the sense of producing a responsiveness to exogenous hormone which differs from that of normal adult females” (page 369).
4. *Dichotomy.* The authors dichotomized the hormonal effects: organizational (“differentiating”) vs. activational. During the prenatal period, testosterone acted to organize tissues so that they respond differently to gonadal hormones in adulthood. In adulthood, the hormones activate tissues organized prenatally. “The embryonic and fetal periods are periods of organization or “differentiation” in the direction of masculinization or feminization. Adulthood, when gonadal hormones are being secreted, is a period of activation; neural tissues are the target organs and mating behavior is brought to expression. Like the genital tracts, the neural tissues

mediating mating behavior respond to androgens or to estrogens depending on the sex of the individual, but again the specificity is not complete” (pages 379–380).

5. *Hormonal effects on the brain.* The authors favored the idea that the brain, like the genital tracts, was permanently masculinized (differentiated) by testosterone. Although their wording carefully leaves open the site of testosterone action (“on the tissues mediating mating behavior”, page 369), the authors clearly favored the view that testosterone or its metabolites acts on the CNS. “We are assuming that testosterone or some metabolite acts on those central nervous tissues in which patterns of sexual behavior are organized” (page 381). (The identification of estradiol as an important metabolite in the brain was an important footnote added later (MacLusky and Naftolin, 1981).
6. *Critical period.* The authors presented evidence for a critical period for testosterone’s action on the brain. Treating females with testosterone postnatally, or in adulthood, did not change their responsiveness to hormones in the long term.
7. *Diverse actions of testosterone.* Although prenatal exposure to testosterone also caused masculinization of the external genitals of females, the effects on mating behavior were dissociated from those on the genitalia because they were not always correlated. This dissociation was not discussed at length by Phoenix et al., but implies that the behavioral effects are not the result of the actions of testosterone on the genitalia, an issue that recurred in later discussions (Beach, 1971).

A critical emphasis of the Phoenix et al. paper was that they were applying and extending a conceptual framework, already developed by Lillie (Lillie, 1916; 1939), Jost (1947; Jost et al., 1973) and others, based on the study of sexual differentiation of the external genitalia and genital tracts. “Attention is directed to the parallel nature of the relationship, on the one hand, between androgens and the differentiation of the genital tracts, and on the other, between androgens and the organization of the neural tissues destined to mediate mating behavior in the adult” (page 369). Specifically, Phoenix et al. argued that the fetal actions of hormones permanently change the substrate (probably neural) on which gonadal hormones act in adulthood, just as they do in the genitalia and genital tracts. By explaining behavioral and genital sexual differentiation in much the same way, the authors provided a heuristically pleasing single framework for explaining all non-gonadal sexual differentiation. The comparison was an invitation to the reader to apply to behavior a host of experimental findings in the period 1916–1959 that indicated that gonadal hormones cause permanent sex differences in tissue differentiation and growth, even though the Phoenix et al. experiments themselves did not measure morphological differentiation and growth. “...When what has been learned from the present investigation is related to what has long been known with respect to the action of androgens in the genital tracts, a concept much broader than that suggested by the older studies emerges” (page 379).

Yet, Phoenix et al. realized that both sexes have significant capacity to show behavior normally seen mostly in the other sex. Thus, behavioral sexual differentiation is incomplete, and the two sexes are each somewhat bisexual. “We suggest... that in the adult this bisexuality is unequal in the neural tissues as it is in the case of the genital tissues. The capacity exists for giving behavioral responses of the opposite sex, but it is variable and, in most mammals that have been studied and in many lower vertebrates as well, it is elicited only with difficulty....” (page 380).

Conceptual frameworks determine experimental designs

The Phoenix et al. theory has dominantly influenced how experiments have been performed ever since. For example, In the brain sexual differentiation literature, the effects of

hormones are not actually measured equally at all life stages. Rather, there has been the tendency to investigate adult hormone and fetal/neonatal hormone effects intensively because those are the focus of the organizational-activational theory. Most investigators now think of adulthood as an extended period in which hormones act on a relatively unchanging neural substrate. Admittedly there are the slow changes related to aging, and some experiences might cause longer lasting changes to the adult neural substrate. But as a rule, if the effects of gonadal hormones are to be tested in adult animals (via manipulations of hormone levels or receptors or synthetic enzymes), the age of gonadal hormone manipulation of adults is not thought to be critical. On the other hand, if the investigator believes that adult hormone levels do not explain a specific sex difference, then the most common manipulation is to administer testosterone to fetal or neonatal females, or to reduce testosterone action in fetal or neonatal males, based on the organizational hypothesis. This focus on two times of life has left some important questions relatively unanswered. For example, does the surge of gonadal hormone secretion at puberty have long-lasting effects similar to the perinatal organizational effects? Recent experiments support that idea (Sisk and Zehr, 2005; Sisk, this volume). Indeed, one might now ask if the pubertal period can be considered a second wave of differentiation of the “tissues that mediate” sexually dimorphic behaviors in adulthood (Ahmed et al., 2008).

The experimental design of Phoenix et al. set a standard for succeeding generations: to measure the permanent effects of gonadal hormones that act during the fetal/neonatal period, compare groups that differ in the levels of fetal hormones but keep the levels of hormones equal across groups at the time of behavioral testing. By equalizing hormone levels across groups, Phoenix et al. made sure that the behavioral effects were measured under conditions conducive to the expression of the behavior (e.g., estradiol and progesterone activate lordosis). Equally importantly, their design eliminated group differences in the activational effects of hormones that might have confounded group differences in organizational effects. That allowed them to attribute the group differences in adult behavior to differences in capacity set up because of the fetal action of testosterone, not to an effect of the fetal treatment with testosterone on subsequent levels of gonadal hormones. This same issue is raised repeatedly in experimental designs in the modern period. For example, in studies that ask if a genetic mutation causes differences in a behavioral system that is influenced by activational effects of gonadal hormones, it remains important to discriminate the effect of the mutation on the neural system from its potential effect on the secretion of hormones (Canastar et al., 2008). For this purpose it is important to test animals under conditions in which group differences in hormonal levels have been eliminated at the time of testing.

Mating behavior and beyond

The sexual differentiation of mating behavior has long been the archetypal example of a sex difference in behavior, and has been studied much more than sex differences in other behaviors. However, when the organizational-activational dichotomy has been applied to the study of other behaviors or neural systems, it fares generally well, although there are notable exceptions (Arnold, 1997). Numerous other articles in the current issue of *Hormones and Behavior* document this conclusion. For example, adult and neonatal manipulations of gonadal hormones have been found to contribute to sex-typical responses to stress and nociceptive stimuli, and sex differences in learning and cognition (Handa et al., 1994; Shors and Miesegaes, 2002; Craft et al., 2004; McCarthy and Konkle, 2005). Not infrequently, however, treating one sex with the hormones of the other sex (i.e., the hormones secreted at higher levels by the other sex) does not completely sex reverse them (e.g., Breedlove and Arnold, 1983b; Mogil et al., 1993). The incomplete sex reversal is usually interpreted not as a failure of the organizational-activational framework, but as a technical issue. If

testosterone given neonatally does not completely masculinize a female, it might be because the hormone was given at a suboptimal time or dose or metabolic form.

Although neonatal or adult manipulations of gonadal hormones (or their receptors or synthesis) are effective in causing at least partial sex reversal of many behavioral and neural phenotypes, it has often not been established, for many phenotypes that differ in males and females, whether the sex difference is completely explained by organizational and activational effects (e.g., by demonstrating that the sex difference is completely sex reversed by giving the hormones of one sex to the other, both fetally/neonatally and in adulthood). Nevertheless, null mutations of estrogen or androgen receptors, which remove both organizational and activational effects of specific gonadal steroid hormones, are effective in eliminating many sex differences in tissue phenotypes (Korach, 1994; Rissman et al., 1999; Ogawa et al., 2004; Juntti et al., 2008).

The organizational-activational dichotomy has also been extended to studies of sex differences outside of the brain. For example, sex differences in the liver are not abolished by gonadectomy of adults (Mode and Gustafsson, 2006; Van Nas et al., 2008), and are thought to be caused, at least in part, by a sex difference in the pattern of growth hormone secretion. The growth hormone pattern, however, appears to be caused by the organizational effects of testosterone on the hypothalamus, which set up the life-long differences in hypophyseal secretions. In other tissues, for example the kidney where there are dramatic sex differences in function, organizational effects of gonadal hormones have rarely been studied, if ever.

Footnotes to the Organizational-Activational Framework

Here we select five groups of findings since 1959 that confirm or extend the organizational-activational dichotomy, or which change our perspective on it.

1. Sex differences in brain structure are explained by the framework

Phoenix et al. speculated that testosterone permanently organizes (masculinizes) the tissues mediating mating behavior, and implied that the changes might be structural. They speculated that the morphological changes would be in the brain, but thought that they would be more modest than the dramatic changes in the genital tracts. Their idea was confirmed by the discovery of morphological sex differences in the brain, beginning in the 1970s (Raisman and Field, 1973; Nottebohm and Arnold, 1976; Gorski et al., 1978; Breedlove and Arnold, 1980; De Vries et al., 1981; Arnold and Gorski, 1984; Simerly et al., 1985). These anatomical sex differences became model systems themselves, useful for investigating the cellular and molecular changes caused by the organizational and activational effects. Surprisingly, some of the sex differences were quite large (e.g., >5 fold sex differences in size of some brain regions), but still not as dramatic as the sex differences in the genital tracts. The experimental approach of Phoenix et al. was used to sex-reverse the volumes of brain regions, or the number or size of cells in those regions (e.g., (Breedlove and Arnold, 1983a,b). Usually, the brain regions were closely implicated in a sex-specific reproductive function such as mating, courtship, or ovulation. Importantly, however, smaller sex differences were also found in other brain regions that are involved in behaviors or functions that are less sexually dimorphic, for example in the thickness of the cerebral cortex (Juraska, 1991,1998). Even in those cases, the organizational-activational dichotomy provided an effective framework to design experiments that supported the idea that testosterone acts in the neonatal males to cause a masculine pattern of brain differentiation (but see McCarthy and Konkle, 2005). The organizational-activational dichotomy has also been applied to studies at the molecular level, and is starting to be applied in bioinformatic studies of the behavior of gene networks (Van Nas et al., 2008).

2. The dichotomy requires a cellular/molecular explanation

If sex steroids have two modes of action, one permanent and the other reversible, what accounts for the difference? On the one hand, the downstream cellular and molecular events mediating organizational and activational effects have not been shown to be dramatically different, and might be quite similar. For example, steroids act to alter synaptic organization of neural circuits throughout life (Nottebohm, 1981; Arnold and Breedlove, 1985; Kurz et al., 1986; Matsumoto et al., 1988; Woolley, 2007). The molecular basis of the organizational-activational dichotomy has been insufficiently addressed, for example in individual studies that contrast the molecular mechanisms mediating organization and activation. The general explanation of the permanence of organizational effects, championed by Phoenix et al., is that when steroid hormones act during the period when the brain is first being put together, the substrate is in a unique configuration that allows external influences to affect tissue organization more profoundly and permanently than at later times in life. Thus, the permanence may not be attributable to the actual genes regulated by testosterone or its metabolites, but on the unusual state of the substrate during early development.

Which cellular processes lead to permanent changes? Studies since the 1980s have suggested that sex steroids probably do not influence the birth of neurons to account for large sex differences in neuron number in brain regions showing prominent sex differences in the number of neurons (e.g., (Breedlove et al., 1983; Cooke et al., 1998), although steroids may modify the rates of neurogenesis elsewhere (Zhang et al., 2008; Ahmed et al., 2008; Galea, 2008). Sex steroids influence the outgrowth of axons and dendrites (Toran-Allerand, 1976), the amount of cell death (Forger, 2006), and regulate the number or type of synapses that a cell makes (Matsumoto et al., 2000; McCarthy, 2008). It is less clear whether gonadal steroids influence other developmental processes such as migration and specification of cell type. The effects of sex steroids on cell death are especially apt as an explanation of the permanence of organizational effects. The developmental overproduction of neurons, followed by an age-limited phase of cell death, is thought to be a once-in-a-lifetime process that does not recur in most brain regions (but see (Ahmed et al., 2008; Galea, 2008). If the cell survives the developmental wave of cell death because of a sex steroid effect, other factors (not gonadal hormones) keep the cell alive and account for the permanence of the sex steroid effect. Moreover, the steroid action during a restricted period of cell death explains the end of one critical period for sex steroid action; once the cells die they cannot be saved from dying by testosterone.

Neurons also go through a developmental period of overproduction of synapses, followed by a limited period of synaptic pruning. In at least one model system, sex steroids can influence the loss of synapses, suggesting a mechanism for selecting specific synapses to be saved as others are lost (Jordan et al., 1992). At the same time, sex steroids are influencing the growth of dendrites permanently, although the permanence of this effect is not yet explained. Once testosterone has organized a dendritic tree, what factors take over and make the change permanent?

The permanence may also result from long-lasting changes in the genome. The recent surge in interest in epigenetic effects provides a new hypothesis to explain permanent sex steroid effects on organization of neural tissues. Do sex steroids alter the chromatin in the region of specific genes, to alter transcription permanently? Recent evidence suggests that histone acetylation is sexually dimorphic in the hypothalamus (Tsai et al., 2009). The permanence of such epigenetic effects is established for other systems (Chang et al., 2006), although it is not always clear how the epigenetic marks on chromatin are maintained. This will be an exciting research frontier in the coming years,

3. Multiple sites of hormone action

Phoenix et al. discussed differentiation of feminine behavior (“feminization”) and of masculine behavior (“masculinization”), again with specific reference to similar processes in the genital tracts. Not mentioned was the idea that the testes secrete a factor that defeminizes the male by inhibiting the differentiation of the Müllerian ducts, established by Jost in the 1940s and 1950s (Jost et al., 1973). Subsequent investigators showed that testosterone, secreted by the fetal and neonatal male (Weisz and Ward, 1980), actively defeminizes the male’s behavior (prevents development of feminine patterns of behavior (Olsen, 1979) in a manner similar to testosterone’s defeminizing effect on female guinea pigs reported by Phoenix et al. Whalen (1968, 1982) emphasized that testosterone’s perinatal role to masculinize and defeminize the male were two separate *orthogonal* processes, since they could be discriminated because they had different critical periods or could be induced independently. Recent studies confirm that masculinization and defeminization have different cellular mechanisms (Schwarz and McCarthy, 2008). Importantly, however, when considering any one site of sex steroid action, or any one cellular or molecular event that is sexually dimorphic, the independence of masculinization and defeminization disappears. If only one sexually dimorphic phenotypic dependent variable is measured, it can vary only along a single continuum of masculine vs. feminine.

4. Neurosteroids

The brain’s ability to make its own steroids could change our perspective on the organizational-activational dichotomy. Sex steroids are produced locally in the brain, both *de novo* and because of local metabolism in the brain of steroid hormones made in the periphery (Schlinger et al., 2001; Baulieu et al., 2001). Despite the accumulation of evidence supporting the importance of local sex steroid synthesis in the brain, that concept has yet to be properly integrated with the classic idea of organizational and activational effects of sex steroids secreted by the gonads. It is often difficult to measure the level of specific hormones at their sites of action in the brain, and plasma levels may not reflect tissue levels (McCarthy and Konkle, 2005). Moreover, it is not clear how changes in plasma levels of hormones dynamically influence the local production of sex steroids in the brain. The factors controlling local synthesis are probably only partially known (Remage-Healey et al., 2008). Classic methods of endocrinology (e.g., ablating the tissue that makes the hormone) are difficult to apply to the brain, thus it will be important to use increasingly sophisticated conditional gene knockout and other methods to manipulate steroid synthesis to understand its role and how that is integrated into a more complete understanding of sexual differentiation.

5. Sex differences in non-reproduction phenotypes and disease

Phoenix et al. were working at a time when sexual differentiation was viewed as a subtopic of the biology of reproduction. The behavioral sex differences that they investigated were essential parts the male’s and female’s sex-specific roles in reproduction. Those phenotypes, like the external genitalia and genital tracts, are among the most sexually dimorphic phenotypes. A major change since 1959 is the increasing interest in sex differences in phenotypes that are not obviously related to reproduction. Males and females show differences in their response to pain and stress, in cognitive tasks, and in a host of diseases that influence the brain. More broadly, nearly all tissues show important sex differences in normal function and disease. For these tissues the functional advantage of a sex difference is often not clear. As an example, why should fat cells function differently in males and females, and why should obesity show sex differences in incidence or progression?

We now realize that some sex differences are not adaptive. It does not make sense that natural selection would favor a greater (or lesser) incidence of a disease in one sex over

another. Instead, these sex differences are likely to be indirect effects of other sex differences that were selected because they were favored in both sexes. For example, males are constrained to have a Y chromosome, even though some Y genes might have pleiotropic effects that are not advantageous in all situations.

Beyond organization and activation: Sex chromosome effects

As organizational-activational dichotomy was increasingly successfully applied to the study of many sex differences, at all levels of analysis from molecular to behavioral, many of us began to be lulled into the expectation that *all* sex differences might be explained by this single theory (as amended by subsequent studies). Few, if any, authors insisted that it explained all sex differences, but hormones became the only factors that were investigated or discussed as proximate signals causing sex differences in the brain. An alternative idea, that the genetic differences between XX and XY cells cause functional sex differences intrinsic to male and female cells, seemed unlikely because XX females in some cases were completely masculine in some phenotypes if they were treated neonatally with testosterone (e.g., Dohler et al., 1984; Nordeen et al., 1985). XY males in which the organizational effects of testosterone were blocked, were completely feminine in some cases (Breedlove and Arnold, 1980, 1983a). There was no need to invoke other factors.

In the period 1989–1995, several discoveries re-awakened an interest in the direct effects of the X and Y genes (O et al., 1988; Renfree and Short, 1988; Beyer et al., 1991; Reisert and Pilgrim, 1991; Burgoyne et al., 1995; Dewing et al., 2003). Sex differences were found before the gonads differentiated, or before plasma levels of testosterone were reported to be sexually dimorphic. Moreover, in at least one model system, the neural circuit for song in songbirds, manipulations of the type of gonad or level of gonadal hormones failed to sex-reverse the phenotype fully (Arnold, 1996, 1997; Wade and Arnold, 1996). Because sex differences in some phenotypes were not explained by organizational or activational effects of gonadal hormones, we turned to a consideration of an alternative but old idea, that genetic differences intrinsic to male and female brain cells might be the origin of some sex differences in phenotype (Arnold, 1996; De Vries et al., 2002). Male and female zygotes have an identical set of autosomes, on average, which comprise about 95% of the genome. Those common genetic factors make males and females quite similar in their function and behavior. The sex chromosomes, comprising the other 5% of the genome, differ in three main ways in the zygote: (1) males alone have Y genes, (2) the two sexes differ in the copy number of X genes (although the sex-specific effect of this difference is largely eliminated by X-inactivation), and (3) females receive a paternal X imprint that is lacking in the male. As the individual develops, however, three other intrinsic genetic sex differences arise that are not caused by gonadal hormones. (1) When X-inactivation occurs, it utilizes some cellular resources only in females, which may make females more vulnerable than males to some genetic mutations or environmental perturbations at early stages of embryonic development (Chen et al., 2008). (2) Female tissues are mosaics. One X chromosome is randomly selected to be transcriptionally silenced in each female cell. Thus, about half of the cells activate the maternal X chromosome and express the maternal X alleles or maternal imprint, and the other half cells express the paternal X alleles or imprint. Mosaic (female) tissues might differ in phenotype from tissues that are not mosaic (male), because they may function better in a range of environments (i.e., different alleles are more adaptive in some environments) or have or have muted susceptibility to diseases involving X genes (Arnold, 2004; Migeon, 2007). (3) In populations of animals, the representation of some alleles may differ between males and females because some gene variants might be less compatible with survival in one sex. Such population effects could lead to average sex differences in phenotype.

Using rodent models, three approaches provide convincing evidence that these differences in XX and XY genomes directly cause sex differences in non-gonadal cells. Such sex differences, called *sex chromosome effects*, could result from any of the differences discussed in the last paragraph, except that there is no allelic variation in inbred mouse strains discussed below. One approach is to interfere directly with the expression of a Y gene to demonstrate that it has a male-specific effect in the brain (Dewing et al., 2006). The testis-determining gene *Sry* is expressed in the adult rodent and human substantia nigra. Antisense oligonucleotides were used to reduce *Sry* expression in the brain of adult rats and mice. Loss of *Sry* led to reduced expression of tyrosine hydroxylase in the dopaminergic cells of the substantia nigra and striatum, and interfered with motor function. This study (Dewing et al., 2006) is the first to identify a Y gene that has a direct effect on brain phenotype. A second approach is study mice lacking the steroidogenic factor 1 (SF-1) gene. These mice lack adrenals and gonads, but survive if they are treated neonatally with corticosteroids and then implanted with adrenal tissue (Grgurevic et al., 2008; Budefeld et al., 2008). This model compares XX and XY mice that never had gonads. SF-1 knockout XY and XX mice differ in body weight and in distribution of specific immunohistochemically defined cells in the preoptic area, bed nucleus of the stria terminalis, and hypothalamus, indicating that sex chromosome complement affects these phenotypes.

The mouse model used most often to date to study sex chromosome effects is the “four core genotypes” (FCG) model, in which gonadal sex and sex chromosome complement are uncoupled (De Vries et al., 2002; Arnold and Burgoyne, 2004; Arnold and Chen, 2009; Arnold, 2009). FCG mice comprise XX and XY gonadal males (XXM and XYM) and XX and XY gonadal females (XXF and XYF). In FCG mice the Y chromosome is deleted for *Sry*, the testis-determining gene, which is then inserted as a transgene onto an autosome. The autosome becomes testis-determining, and the sex chromosomes are irrelevant to the gonadal sex (testes vs. ovaries) of the animal. This model allows testing of organizational and activational effects of hormones, but more importantly also can be used to test for group differences in XX vs. XY mice that have the same type of gonads. Such differences between XXM and XYM, or between XXF and XYF, are attributed to the differential effects of an XX vs. XY genome. Finally, the model tests for interactions of hormonal and sex chromosome effects, for example if testosterone has different effects in XX vs. XY mice (Arnold and Chen, 2009).

The FCG model was first used to examine possible sex chromosome effects on several brain and behavioral traits long known to show sex differences (De Vries et al., 2002). In each case, previous work had demonstrated that these sexual dimorphisms were caused by organizational and/or activational effects of gonadal steroids. Among the phenotypes measured were male copulatory behavior, and morphological sex differences in vasopressin fibers in the lateral septum, in the spinal nucleus of the bulbocavernosus (SNB), and the hypothalamic anteroventral periventricular nucleus (AVPV). The FCG mice were gonadectomized as adults and treated equally with testosterone (mirroring the classic methods of Phoenix et al.), so that group differences were not attributable to group differences in activational effects of sex steroids. For these phenotypes, studies of FCG mice confirmed in all cases that the sexually dimorphic phenotype differed in mice that developed with testes vs. ovaries (i.e., were caused by organizational effects revealed as differences between in XXM vs. XXF, and in XYM vs. XYF). For most of the phenotypes, there were no differences between XX and XY mice that had the same gonadal sex. The same results were obtained in studies of sex differences in thickness of the cerebral cortex, and in progesterone receptor expression in the preoptic nucleus of the hypothalamus in neonates (Markham et al., 2003; Wagner et al., 2004). Thus, the organizational-activational

framework accounted completely for the majority of the classic sex differences first studied in the FCG model.

One exception was that septal vasopressin, in addition to being sexually differentiated by organizational and activational effects of sex steroids, shows a small difference between XX and XY mice, with XY mice of either sex showing greater vasopressin fiber density than XX mice of the same sex (De Vries et al., 2002; Gatewood et al., 2006). Thus, for this phenotype it appears that sex chromosome factors might sum with hormonal effects to produce sex differences.

Further studies of FCG mice have uncovered convincing new evidence that sex chromosome complement has an important impact on sexually dimorphic phenotypes, in some cases producing large sex chromosome effects (Arnold and Chen, 2009; Arnold, 2009). Based on these studies as a group and those mentioned from other approaches reviewed above, there is little doubt that XX and XY cells are intrinsically different, not just because of organizational and activational effects of gonadal hormones. The following is a list of examples.

- A. When mesencephalic cells are dissociated and cultured from mouse embryos 14.5 days after coitus, the number of dopaminergic neurons that differentiate (I.e., those expressing tyrosine hydroxylase) is greater in cultures from XY mice of either gonadal sex, compared to XX mice (Carruth et al., 2002). This study confirms that XX and XY mesencephalic cells have different properties that are not caused by effects of gonadal steroids (Beyer et al., 1991; Reisert and Pilgrim, 1991).
- B. When FCG mice are gonadectomized as adults and treated equally with testosterone, the XY gonadal females are more aggressive than XX females, as evidenced by their more frequent attack of a male intruder mouse in the home cage) than XX females, whereas XX and XY males are equally aggressive. XX and XY females also show differences in measures of parenting behaviors and social interactions (Gatewood et al., 2006; McPhie-Lalmansingh et al., 2008).
- C. In FCG mice that are gonadectomized in adulthood, the expression of prodynorphin mRNA in the striatum is higher in XX mice than XY mice (Chen et al., 2009). This gene encodes the precursors of the dynorphin peptides that are ligands of the kappa opioid receptor. Based on the idea that the robust difference between XX and XY prodynorphin expression might influence the mouse's response to nociceptive stimuli, we tested gonadectomized adult and gonadally intact neonatal mice in several tests of thermal and chemical nociception (Gioiosa et al., 2008a; Gioiosa et al., 2008b). In all cases, the XX mice showed greater or faster responses than XY mice, irrespective of gonadal sex. The greater responsiveness of adult gonadectomized XX mice suggests that sex chromosome complement contributes to sex differences in response to nociceptive stimuli.
- D. XX and XY mice also differ in tests of habit formation that are potentially relevant to addiction (Quinn et al., 2007). Human females are reported to increase usage of some drugs more quickly than males, to the point of addiction. Mice learn a task if they receive food reward, which can progress to a "habit" after continued conditioning. A habit is relatively insensitive to the contingencies of reinforcement such as the value of the reinforcer, in contrast to the initial stages of conditioning when reward value is important to the performance of the task. In an experiment in which FCG mice were trained to respond to food reward, XX mice of either gonadal sex progressed to the level of habitual responding (continued responding despite devaluation of the reward) more quickly than XY mice of either gonadal sex.

(Quinn et al., 2007). Thus, sex chromosome complement may influence the rate at which mice develop a habit.

- E. Neural tube closure defects influence human female neonates more than males. In some mouse models of neural tube defects, the sexes also differ in the effects of the mutation that interferes with tube closure. In mice with a null mutation of the p53 gene, female embryos develop more anencephaly and exencephaly than do male embryos, and most females lacking p53 die by the day of birth. The sex difference is caused by XX vs. XY differences in the genome, not by gonadal hormones, as was demonstrated in FCG mice (Chen et al., 2008).
- F. The incidence and progression of autoimmune diseases is sexually dimorphic. Females are more affected than males by multiple sclerosis (MS) and systemic lupus erythematosus (SLE). In mouse models of these diseases, females are often more susceptible than males. The mouse models involve treating mice with an antigen and/or adjuvant that triggers an autoimmune response with properties similar to MS or SLE. When gonadectomized adult FCG mice are used in these mouse models, XX mice fare much worse than XY mice. The progression of the MS-like disease is faster in XX than XY mice of either sex, and in the SLE model XX mice die faster than XY (Smith-Bouvier et al., 2008). Although organizational and activational effects of gonadal hormones also explain some sex differences in the MS-like model (Voskuhl and Palaszynski, 2001; Voskuhl, 2009), it appears that sex chromosome complement also plays a role.

An important issue in comparing FCG mice is whether the sex chromosome effects, which are difference in XX vs. XY groups, could themselves be caused by group differences in the levels of gonadal hormones. In all cases mentioned, the effects are measured in the absence of gonads, so the group differences are not caused by activational effects. It is conceivable, however, that XX and XY males, or XX and XY females, might have received different exposure to gonadal secretions prior to adult gonadectomy. On balance such differences appear to be unlikely, because the mice of the same gonadal sex appear to be equally masculinized (or not) on a number other variables (De Vries et al., 2002; Markham et al., 2003; Wagner et al., 2004; McPhie-Lalmansingh et al., 2008), and because in some cases there are no organizational effects as measured in the FCG model itself (Gioiosa et al., 2008a; Arnold and Chen, 2009). Ultimately, the mechanisms mediating the effects can be established when the gene(s) mediating the effects are identified.

The long term goal of studies of sex chromosome effects is to find the genes responsible, and their mechanisms of action. Several X and Y genes appear to contribute to sex chromosome effects. As indicated above, the Y-linked *Sry* gene has been shown to have male-specific effects on the substantia nigra and striatum. The sex chromosome effect on prodynorphin expression is explained by the difference in number of X chromosomes, since XO mice have similar prodynorphin expression as XY mice, which is less than XX (Chen et al., 2009). Similarly, the sex chromosome effect on neural tube closure in p53-deficient mice is an X-linked effect based on similar evidence from mice with different numbers of X and Y chromosomes (Chen et al., 2008). The X effects could either be differences in the expressed dose of X genes that escape X-inactivation (i.e., higher expression in XX than XY), or the result of XX vs. XY differences in the expression of X genes that are parentally imprinted (different levels of expression in XX vs. XY because only XX mice receive a paternal X imprint).

A unified theory of the origins of sex differences in all tissues

Building on the foundation provided by Phoenix et al. and other major figures in endocrinology and genetics of sexual differentiation (Lillie, 1939; Jost et al., 1973;

Goodfellow and Lovell-Badge, 1993), we can update a general model for the origin of sex differences in tissue phenotype (Arnold, 2002, 2004)(Figure 1). We propose the following model:

All ontogenetic sex differences in phenotype derive from the differences in the effects of sex chromosome genes, which are the only factors that differ, on average, in the male and female zygote. A subset of X and Y genes represent the primary sex-specific factors causing sex differences in development and adult phenotype. Primary among these is *Sry* because it controls sexual differentiation of the gonads, and therefore sets up life-long sex differences in secretion of gonadal hormones. These hormones, especially testosterone and estradiol, act throughout the body in an organizational (long lasting or permanent) and an activational (reversible) fashion at different times of life, to cause most known sex differences in phenotype, including sex differences in susceptibility to and progression of diseases. In addition to *Sry*, however, various X and Y genes have differential effects on male and female cells because of the constitutive sex differences in the copy number and/or parental imprint on these genes. Various sex-specific factors interact, acting synergistically or counteracting each other or otherwise conditioning the effects of each other. Thus, XX and XY cells are different prior to the secretion of gonadal hormones, and gonadal hormones affect XX and XY cells unequally.

In contrast to the general model that operated in the period from 1916 to the late 1980s, the unified model shifts the emphasis away from the gonadal hormones as the sole agents that act on non-gonadal tissues to cause sex differences in phenotype. Instead, the gonadal hormones are seen as most important among a variety of secondary factors that are downstream of the primary sex-specific effects of X and Y genes. The primacy of the X and Y genes stems from the fact that they are the only factors in the zygote that give rise ultimately to sex differences in phenotype. Logically, *Sry* and other Y and X genes should be now ranked as the primary (but not necessarily the most proximate) agents of sexual differentiation, since their role in this regard derives from the constitutive difference in XX and XY genomes. Although *Sry* is so far the only X or Y gene identified to play this role, recent evidence makes it clear that X genes and possibly other Y genes must also be primary in their sex-specific effects that lead to sex difference in phenotype (Chen et al., 2008, 2009). Steroid hormones secreted by the gonads retain a special place among the secondary proximate factors (those not sexually dimorphic in the zygote) causing sex differences, because of their widespread and dominant effects on sexual phenotype of many tissues. But gonadal secretions only make sense as important factors because sex differences in the levels of gonadal secretions can be demonstrated to derive directly from (be downstream of) the effects of *Sry*, one of the primary X or Y genes that are agents of sexual differentiation encoded by the sex chromosomes. Thus, to explain the process of sexual differentiation of any phenotype, it remains critical to identify the sex chromosome factors that cause the sex difference in the proximate factors that directly cause the sex difference in phenotype.

The proposed model is unified because it applies equally to all tissues and sex differences in phenotype. The old dogmatic separation of the gonads and other tissues is gone. Previously, sex differentiation of the gonads was seen as “genetic”, and sexual differentiation of other tissues was seen as “hormonal”. This is an old dichotomy established in the first half of the 20th century (for example, see Lillie, 1939). Today, we realize that there is no reason to expect that any tissue is immune to the effects of any of these sex-specific factors. The re-unification of gonads and other tissues is an attractive conceptual simplification. In addition, it is important to integrate investigations of direct genetic and hormonal factors and think about their interactions in the single conceptual framework.

The last sentence of the unified model deals with a question that has been studied relatively little, and therefore this sentence admittedly goes beyond hard evidence at the present time. To date, sex chromosome effects have been reported under conditions in which the activational effects of hormones are eliminated. Relatively little information is available on how gonadal hormones (either organizational or activational effects) affect the phenotypes in which sex chromosome effects occur. Do hormones swamp out the sex chromosome effects, which would be possible only if the hormones have differential effects on XX and XY cells? Do the hormones enhance some sex chromosome effects, so that the direct genetic and hormonal effects sum to produce sex differences in phenotype? Or, do hormonal and direct genetic effects counteract each other, reducing sex differences caused by the other (De Vries, 2004, 2005; McCarthy and Konkle, 2005)? What molecular/cellular mechanisms mediate the sex chromosome and hormonal effects, and account for their interactions? These questions about interacting hormonal and sex chromosome effects should be asked, both with regard to activational and organizational effects of gonadal hormones. These are major questions to be investigated in the future.

Because sex differences are caused directly by both endocrine and cell-autonomous or tissue-autonomous genetic effects, the investigation of sex differences in the future will require an expanded toolkit incorporating classic endocrine methods to manipulate hormone synthesis and action, modern molecular genetic methods to alter hormone action in a cell type-specific manner (Wintermantel et al., 2006; Monks et al., 2007), as well as methods to manipulate the copy number and expression of X and Y genes that underlie constitutive genetic differences in XX and XY cells. Students of sexual differentiation, as always, must develop an appreciation for how gonadal hormones cause sex differences, but also need to expand their conceptual horizons to include an understanding of the sex chromosomes and the direct effects of genes encoded on these chromosomes, and the interaction of these genes with the rest of the genome.

An interesting question is whether sex chromosome effects are organizational or activational. Are they permanent, caused by sex chromosome effects early in ontogeny, or are they reversible? For *Sry*, the only Y gene that has been shown to have direct male-specific effects on the brain (Dewing et al., 2006), the effects are at least partly reversible, since blocking *Sry* expression in the adult brain causes reversible changes in dopamine systems and behavior.

Another interesting question is why most sex differences are caused by gonadal hormones (Arnold, 2002, 2004). Sex differences are favored in evolution when a phenotype is adaptive in one sex more than the other. Under those conditions, and to establish the sex difference, the development or expression of the trait comes under the influence of a sex-biased or sex-specific factor. Sex-biased factors that evolve control of phenotypes most often will be those that are widespread (present in many cell types) or for which the smallest number of mutations are required for that factor to influence the phenotype. Gonadal hormones might repeatedly evolve a controlling role since they are present throughout the body and are sex-biased at many life stages. Moreover, a few mutations (for example, to increase expression of the steroid receptor in the tissues controlling the phenotype) might be necessary to evolve a sexual dimorphism. Although the sex chromosome genes are also present in each cell, we have little information about the kinds of cellular processes that they can influence. Thus, further information on the X and Y genes may be necessary to explain why gonadal hormones have become the dominant class of sex-biased factors that have proximate influences causing sex differences in phenotype.

In summary, a central concept of the unified theory is that various X and Y genes are now considered primary causal agents of sexual differentiation because these genes are

differentially represented in the male and female genome. Thus, a full explanation of the forces causing sex differences in phenotype must include identification of those X or Y genes. To date, only *Sry* is known to play this primary role, but even for *Sry* the sex specific effects on the brain are both direct and indirect, mediated by the differential effects of testicular and ovarian secretions that are set up by *Sry* expression in the embryonic testis, and by *Sry*'s direct effect on the brain. Although other X or Y genes also have direct sex-specific effects, it seems unlikely that they will be seen as co-equal with *Sry* because of its role in gonadal differentiation leading to dominant organizational and activational effects of gonadal hormones.

Epilogue: A personal note

In many ways, the organizational-activational dichotomy has provided the central theoretical framework for much of the work of my lab over the last 30 years or more. I began working on sex differences in the brain and behavior about 16 years after the publication of the Phoenix et al. paper. In 1975 Fernando Nottebohm and I accidentally discovered large morphological sex differences in the neural circuit for song in Passerine birds (Nottebohm and Arnold, 1976). Once I set up in my own lab, we began to use the song circuit and the spinal nucleus of the bulbosacrus (Breedlove and Arnold, 1980) to study the cellular effects of androgens and estrogens in the CNS. Among the questions that we wanted to answer, at the cellular level instead of at the behavioral level that others had used before us, were: (1) What are the differences between organizational and activational effects (Arnold and Breedlove, 1985)? (2) What cellular events define the critical period for hormone action (i.e., what is critical about the critical period)? (3) What accounts for the permanence of the organizational effects? I hope that readers will forgive me for overemphasizing our own work by citing here some of our studies from a couple of decades ago concerning those questions.

To investigate the origin of sex differences in the neural song circuit in zebra finches, we modeled our experimental manipulations in birds on those that had supported the organizational theory in mammals (Arnold and Schlinger, 1993). We attempted to interfere with synthesis or action of gonadal steroids in young males, and found that these manipulations had little effect on the development of a masculine neural circuit in males (Arnold, 1997). Yet, because of the dominance of the organizational-activational framework, we were originally quite reluctant to consider the idea that the sex differences are not caused by gonadal hormones. Eventually, however, we investigated sex differences caused by direct sex chromosome effects, in part because of emerging molecular genetic evidence that the X and Y chromosomes are expected to have unbalanced effects in XX and XY cells. In general, molecular geneticists who study the sex chromosomes seem less skeptical of the idea that X and Y genes directly cause sex differences in phenotypes, compared with behavioral neuroendocrinologists who, like me, were thinking mostly about hormones, and were impressed by the powerful organizational and activational effects of hormones. It is a tribute to the Phoenix et al. (1959) paper, and others that followed, that our thinking was dominantly focused on hormones.

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Figure 1.

Contrast between the predominant 20th Century model to explain sex differences in the phenotype of tissues, with a revised model. In the 20th Century model, the sexual differentiation of the gonads is ascribed to the male-specific effect of the Y-linked gene *Sry*. Once the gonads have differentiated, they secrete different sex steroid hormones. Based on the organizational-activational framework of Phoenix et al., (1959), the testicular hormones testosterone and Müllerian inhibiting hormone (MIH) acts on diverse tissues (e.g., genital tracts and brain) in the fetal/neonatal male to cause masculine patterns of development resulting in permanently sexually differentiated substrates. Later in life, ovarian and testicular hormones act differentially on those substrates to create further sex differences in phenotype. In contrast, the unified model recognizes that *Sry* and other (to be identified) X and Y genes occupy the same primary logical level because they are all unequally encoded by the sex chromosomes in males and females. Some X and X genes act in a sex-specific manner, on the gonads and other tissues, to cause sex differences in XX and XY cells. *Sry* plays a dominant role by setting up the lifelong sex difference in secretion of gonadal hormones, which have organizational and activational effects on the brain and other tissues. Because of the independent sex differences in sex chromosome genes, and in hormonal secretions, the various sex-specific factors interact in one of several ways. Their effects are synergistic (as for example when Y factors and testicular testosterone both push the male's tissues to function differently than in females), or they counteract each other to reduce sex differences (for example when the female-specific process of X-inactivation shuts down one X chromosome in each female cell to counteract the female bias in X gene expression that would otherwise occur). With minor modification the schema shown here can apply equally to birds or other groups that have a constitutive sexual imbalance of sex chromosome genes, by substituting species-appropriate sex determining gene(s) for *Sry*.