UNDERSTANDING THE EFFECTS OF SEX CHROMOSOMES AND SEX HORMONES ON SEX DIFFERENCES

by

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ABSTRACT

Biological factors that determine sex and drive sexual differentiation have long been established in humans and other mammalian species. Yet there is a limited understanding of the mechanisms by which these factors give rise to sex-biased outcomes in health and disease. Growing evidence for sex differences in preclinical and clinical studies has led to research funding regulations that require clinical studies and drug trials to account for sex as a biological variable. This new wave of interest in sex differences is focused on three aspects: First, what factors show quantitative sex differences at the molecular and cellular levels, and how are they regulated, over the course of a lifetime, by the classic sex-biasing factors, i.e., genes encoded on the sex chromosomes and sex steroid hormones secreted by gonads. Second, can we utilize knowledge of sex differences (e.g., sex-biased gene expression) gained from studying model animals to generate and test hypotheses regarding these sex-driven variables in a multitude of biological functions of interest. Third, can we translate basic knowledge of sex differences into new therapeutic targets/strategies that alleviate sex-biased outcomes in common diseases, developing sex-specific treatments or extending sex-biased protection from one sex to the other. In this thesis, I will first review the fundamental causes of sex differences and the classical strategies used to study sex-biased phenotypes. Next, I will introduce existing mice models (i.e., the “Four-Core-Genotype”, XY* and Sex Chromosome Trisomy models) that aid in delineating the effects of sex hormones and sex chromosomes on sex differences. Finally, I will highlight the insights gained from recent studies using these mouse models and discuss the need for a “systems approach” in future studies of sex differences. (276 words.)

Thesis Supervisor: David C. Page
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Rationale

Why study sex differences?

Recent NIH policies mandate the consideration of sex as a biological variable in vertebrate animals and human studies (National Institutes of Health, 2015). The concern stems from the prevalence of clinical research conducted on an almost homogenous white male population while applying these results in medical practice to males and females of all races (Mastroianni et al., 1994). To generate clinical results that are valid for the wider population, it is necessary to include both sexes and individuals of diverse geographic origins in clinical studies and drug trials (Blauwet et al., 2007). Perhaps more importantly, sex should be investigated as a primary variable in basic biological research that relies on animal models. Understanding the sex-dependent differences and similarities in fundamental biological processes will lead to future translational research in prevention, diagnostics and therapeutic interventions for sex-biased diseases (Wizemann & Pardue, 2001).

How do we study sex differences?

Sex is among the first phenotypes studied in any model organism. Studies of sex determination, sex differentiation of the reproductive tract and brain, and the extensive effects of gonadal steroid hormones all fall into the broader category of sex differences research. Essentially, all sex difference investigations in animals rely on manipulating factors that drive sexual dimorphism – gonadal steroid hormones and sex chromosomes – to study their effects. In many animal models, direct interventions that include surgical removal of gonads, hormone replacement, or genetic manipulation of sex chromosomal genes allow for the controlled study of downstream effects in a laboratory setting. In humans, however, researchers must rely on the occurrences of spontaneous mutations to eliminate or sex-reverse various sex-biasing factors. Comparing these cases to age-matched controls allows the researcher to infer the normal functions of these factors in humans.

What factors determine sex?

The source of all sex differences begins with the composition of sex chromosomes in the zygote. In this beginning stage of life, the sex chromosome complementation, commonly XX or XY, is the most prominent difference between zygotes that will develop into females and males, respectively. The X and Y sex chromosomes are heteromorphic, resulting in the imbalance in genetic information carried in males (1X+1Y) and females (2X). Specifically, the presence of the Sex-determining Region Y (*SRY*) gene in males initiates the differentiation of the gonadal ridges into testes while the absence of *SRY* in females initiates development of ovaries. These gonads then secrete gonadal hormones (testosterone from testes; estrogen and progesterone from ovaries) in a sex-specific pattern that sets up different hormonal profiles in males and females over a lifetime. Outside of the gonads, *SRY* is also expressed in the brain and other tissues that contribute to male-biased phenotypes (Ely et al., 2010). Other genes on the Y, though mostly expressed in the testes, may also contribute to non-gonadal sex differences. Further sources for sex differences include genes that escape X-inactivation, differential parental imprinting of genes on the X chromosome during spermatogenesis and oogenesis, and the X- vs. Y-related effects of epigenetic regulation on autosomal genes.

Section 1: The Origin of Sex Differences

1.1 Three types of sex-differential phenotypes

Phenotypes that differ between the sexes can be grouped into three non-exclusive types (McCarthy et al., 2012). The first type, termed *sex dimorphisms*, refers to phenotypes that are qualitatively distinct in males and females (e.g., reproductive traits) whereby one form appears only in males (e.g., testes) and another form only in females (e.g., ovaries). Hermaphrodites and gynandromorphs are interesting exceptions where both traits are present in one organism. The second type, commonly referred to as *sex differences*, encompasses phenotypes that fall along a measurable range but the distribution of the phenotypic measurements for the male population differs from that of the female population (e.g., height). The third type, *latent sex differences*, refers to sex-biased phenotypes that are revealed under certain conditions. These are phenotypes that start out the same in males and females but diverge in response to a perturbation (e.g., chemical exposure, genetic manipulation, environmental or psychological stress). Under normal circumstances, such a phenotype may be driven by two or more upstream factors that compensate (i.e., in an additive manner) or negate (i.e., opposing effects that cancel out) one another.

1.2 Classic sex-biasing factors and their effects

The effects of the classic sex-biasing factors – gonadal steroid hormones and sex chromosome complement – have been classified into three categories (Arnold, 2009a). A sex-biased phenotype could be driven by (1) *activational effects* of gonadal steroid hormones, (2) *organizational effects* of gonadal steroid hormones, and/or (3) *sex chromosome* effects. Prior to the differentiation of the gonads, the sex chromosome complement of the developing embryo is the only sex-biasing factor that differs between the sexes. Once the gonads are differentiated and begin to secrete testicular or ovarian hormones, sex-specific profiles of gonadal steroid hormones are gradually established in males and females. From this point forward, both gonadal steroid hormones and sex chromosomal genes could exert (non-exclusive) effects on cells and tissues across the body.

The three categories of sex-biasing effects are useful guides to the study of sex differences as each category is best revealed by specific experimental strategies and outcomes. By definition, a sex difference driven by *activational effects* of gonadal steroid hormones will cease to show a difference when gonadectomy is performed in the male and female animals. Furthermore, the sex difference can be rescued by treatment with one, or a combination, of the gonadal steroid hormones that circulate in the bloodstream of males or females. In essence, these sex differences are only observed when the effective sex hormone is present; they subside or disappear when the hormone is withdrawn.

The “organizational effects” of gonadal steroid hormones drive sex differences in adulthood that *persist* after gonadectomy, i.e., the acute withdrawal of sex hormones has no effect on the sex difference. These sex differences are hypothesized to be due to gonadal steroid hormones acting

in early stages of development, resulting in a long-lasting and perhaps even permanent sex difference that is still observed later in adulthood (Phoenix et al., 1959). Examples include sexual differentiation of the external and internal genitals (Jost et al., 1973), and sexual dimorphisms in the brain and behavior (Arnold & Gorski, 1984; Breedlove & Arnold, 1980, 1983b; Döhler et al., 1984; Nordeen et al., 1985).

Finally, sex differences due to sex chromosomal effects can be observed at embryonic stages before the differentiation of sex-specific gonads (Burgoyne et al., 1995; O et al., 1988; Reisert & Pilgrim, 1991) or just before testosterone levels significantly diverge between male and female fetuses (Beyer et al., 1991; Dewing et al., 2003). These observations are potentially explained by differences in the numbers or functions of genes found on the sex chromosomes, carried within all cells.

Section 2: Strategies to Study Sex-Biasing Factors

Over the past century, a relatively standardized strategy has been established to investigate which of the classic sex-biasing factor(s) is responsible for sex differences in the phenotype of normal tissues (Arnold, 2009b; Becker et al., 2005). The same strategy, in theory, could also be utilized to study other factors that induce, or protect against, sex-biased disease. The general strategy takes the researcher through a sequence of four experiments (of increasing complexity) to test whether each of the classic sex-biasing factors exerts a causal effect on a sex difference of interest. In this section, I will outline this experimental approach in detail to help us understand its historical and current impact on sex difference studies.

2.1 Establishing a sex difference in the phenotype of interest

Quantifying an observable sex difference is an empirical endeavor. Only for the most obvious differences between males and females (e.g., reproductive organs, height) would a simple two-group study involving a male group and a female group suffice to establish a sex difference. To accurately quantify more subtle phenotypes or sex differences found at the cellular and molecular level, researchers have to employ the appropriate methods of measurement and keep track of potential confounding parameters at the time of measurement. Parameters to consider include: the age at which the animals are tested, the time of day of the testing, the environment in which the animal is raised, the type of assay used to measure the phenotype of interest, *etc*. A natural confounder of sex differential phenotypes in humans and animals is the fluctuation of ovarian steroid hormones during the ovarian cycle. One strategy to overcome this confounder is to use a multi-group experimental design to compare the male group to different groups of females, one for each of the days/stages in an estrous cycle (Becker et al., 1982). Moreover, one should also take into account other variables that co-vary with the ovarian cycle and measure each of these variables individually. These covariates could reveal related sex-biasing factors or help to build a better mathematical model of a sex-biased phenotype (e.g., circadian feeding patterns). For the study of subtle sex differences, it is even more critical to first establish a robust sex difference in gonad-intact, wild type animals before proceeding to investigate its causes and effects.

2.2 Testing for the "activational effects" of gonadal steroids hormones

In laboratory animals. After establishing a robust sex difference, we can proceed to test if the sex difference is due to the presence of a particular gonadal steroid at the time of phenotypic measurement. This test ("classic endocrine experiment") involves two steps: endogenous endocrine ablation and exogenous hormonal treatment. In the first step, testes and ovaries are removed from males and females, respectively, and controls undergo sham operations where incisions are made but gonads are not removed. The sex difference is then re-assayed after the operation over a period of days or weeks, allowing the acute effects of gonadal steroids to dissipate over time. If gonadectomy abolishes the sex difference, via a change in the phenotype of interest in one sex (or both), we can conclude that *the gonads* are necessary for the sex-biased phenotype in that sex (or both sexes). Other factors may be indirectly manipulated by

gonadectomy but they would have to be tested individually if we suspect that they are contributing to the sex difference.

To test for the influence of gonadal steroids hormones, we proceed with the second step of the endocrine experiment by administering a specific gonadal hormone to the gonadectomized animals (controls receive a placebo treatment) and re-assay the phenotype of interest. Using a three-group design (gonadectomized animals given gonadal hormone, vs. gonadectomized given placebo, vs. gonad-intact controls) we test if the gonadal hormone rescues the sex difference in gonadectomized animals. For example, when testosterone treatment in castrated male rats rescued penile erections to levels measured in gonad-intact males, we can conclude that testosterone has an acute "activational effect" on the trait (Bradshaw et al., 1981). Some empirical considerations for this step of the experiment include: which gonadal hormone to administer (testosterone or estradiol/progesterone, alone or in some combination), the appropriate dosage to achieve physiological range (as opposed to pharmacological dosages), and the appropriate time-points for post-treatment assays. Alternative methods to the classic two-step experiment include using clinical drugs to inhibit gonadal hormone receptors, or to block the synthesis of the steroid hormone, or the generation of null mutants for specific steroid receptors in animal models (Couse & Korach, 1999).

In humans. Studies in humans face additional hurdles compared to those in animal models. Researchers cannot remove gonads in humans for experimental purposes but they can observe the effects of surgical removal of the gonads in surgical menopause (Sherwin, 1988) and androgen deprivation treatment in men with prostate cancer (Salminen et al., 2003), or study the effects of hormone replacement therapy in postmenopausal women (Duff & Hampson, 2000; Shaywitz et al., 1999). However, age is a confounding factor in many such clinical studies because most patients undergoing these treatments are older. To study the acute effects of gonadal steroids in healthy individuals, researchers could temporarily inhibit gonadal steroid receptors via drugs followed by hormonal treatment (Snyder et al., 2016). These strategies mimic the classic endocrine experiment in animal models but rely on a reversible ablation of sex hormones. Additional variables to take into account include prior environmental exposures, age, geographic ancestry, and the menstrual cycle.

2.3 Manipulating prenatal gonadal hormone levels to test for "organizational effects"

In laboratory animals. In cases where gonadectomy in adulthood does not completely eliminate a sex difference in a phenotype of interest, or in cases where gonadectomized males and females treated with the same gonadal steroid hormone begin to manifest a sex difference, we conclude that the acute effects of gonadal steroid hormones are absent. Instead, we would hypothesize that gonadal steroid exposure at an earlier timepoint in development caused permanent sex-biased differentiation in developing tissues/organs. These structural differences then manifest as sex-biased phenotypes in adulthood. Experimentally, we should test whether removing, or sex-reversing, the gonadal steroid hormonal exposures in prenatal animals eliminates or reverses a sex difference later in adulthood.

There has been a greater focus, in the literature, on the role of testosterone in sexual differentiation during early development. Evidence for the roles of ovarian secretions in feminizing development of the brain has been reported but the effects are subtle and seem to act in a later developmental period compared to the masculinizing effects of testosterone (Fitch & Denenberg, 1998). To manipulate (i.e., sex-reverse) testosterone levels in prenatal animals, two approaches are often used: (1) increase testosterone exposure in prenatal females, or (2) reduce testosterone levels in prenatal males. In the first approach, prenatal females are exposed to increased testosterone exposure via subcutaneous injection of testosterone into pregnant female rats (Breedlove & Arnold, 1983a) and guinea pigs (Phoenix et al., 1959). Controls are female and male littermates from pregnant females who received a placebo treatment. The sex difference is then assayed in the testosterone- and placebo-treated animals when they reach adulthood. If testosterone-treated females resemble control males, we can then conclude that testosterone exerted an "organizational effect" in these females.

In the alternative approach, testosterone levels are reduced in prenatal males by administering an androgen receptor blocker (e.g., flutamide) to pregnant females (Breedlove & Arnold, 1983b). After birth, male pups are gonadectomized (controls are sham operated) and the sex difference is measured in adulthood. If removing testicular secretions during the fetal and postnatal periods prevented masculinization of the phenotype, we can conclude that testosterone elicits an "organizational effect" on males during early development. Furthermore, if postnatal testosterone treatment in gonadectomized male pups rescued the masculine phenotype, we can conclude that the sex difference is sensitive to testosterone during the postnatal period as well.

In sum, we can draw conclusions regarding the long-lasting "organizational effects" of gonadal steroid hormone(s) if the sex difference in adulthood is eliminated after manipulating the animal's prenatal gonadal hormone exposure. However, two issues in these experiments invite alternative hypotheses regarding the sex difference. First, testosterone does not completely masculinize females, and second, anti-steroid treatment followed by postnatal castration does not completely de-masculinize males. Furthermore, we cannot rule out other sex-biasing factors (e.g., sex chromosomal genes) that act independently, or in parallel to, gonadal hormones during sex differentiation.

In humans. Females with congenital adrenal hyperplasia (CAH) caused by recessive mutations in 21-hydroxylase or 11-hydroxylase are exposed *in utero* to excessive levels of androgens produced by the adrenal glands. Symptoms in infants include ambiguous genitalia in girls and enlarged penis in boys, and treatment with cortisone replacement therapy within the first few weeks after birth returns androgens back to normal levels. This provides a model in humans to study the effects of increased prenatal androgen on sex differentiation (Berenbaum et al., 2000; Zucker et al., 1996). In XY individuals with androgen insensitivity syndrome (AIS), caused by deletion or mutation in the androgen receptor, tissues are unable to respond to androgens and undergo female differentiation (e.g., showing incomplete or partial female genitalia). These individuals could be studied to gain insight into the role of androgens in sexual differentiation in comparison to healthy males (Hines et al., 2003). However, some AIS individuals are raised as females and

undergo estrogen supplementation beginning in childhood; the effects of additional estrogen exposure in childhood could confound the effects of AIS.

2.4 Assessing the effects of sex chromosome complement in sex difference

Some sex differences cannot be explained by the effects of gonadal steroid hormones. In some cases, the sex difference in phenotype persists despite manipulations of gonadal steroid exposure in prenatal or adult animals. For example, genetic female zebra finches treated with an inhibitor of estrogen synthesis developed both functional testicular and ovarian tissues but retained a feminine neural circuit for song (Wade & Arnold, 1996). In other cases, sex differences in phenotypes arise before the differentiation of the gonads. Examples of such traits include: differences in the rate of development of male and female embryos observed prior to gonadal differentiation (Burgoyne et al., 1995; Erickson, 1997); in the tammar wallaby, sex-specific body structures such as pouch and scrotum differentiate before gonads (O et al., 1988); neurons dissociated from male or female embryonic rat brain (before gonadal secretions are present) show sex-dimorphic growth rates when cultured in identical conditions (Beyer et al., 1991; Beyer, Eusterschulte, et al., 1992; Beyer, Kolbinger, et al., 1992); X-linked genes show differential expression in male and female primates, resulting in sex differential color vision (Dulai et al., 1999).

Sex chromosomes could potentially exert sex differentiating effects from the earliest stages of embryogenesis. This form of sex differentiation is well established in various tissues in *Drosophila melanogaster* and *Caenorhabditis elegans* (Cline & Meyer, 1996) and in the sex differentiation of the mammalian gonads (Capel, 1998). To test for sex chromosomal effects, one should manipulate the dosage of X and/or Y genes (including allelic variants of these genes) in animals with a constant, or zero, level of gonadal steroid hormones. In practice, achieving a perfectly male or female gonadal hormone profile in an XX or XY mouse is challenging, as shown by the incomplete masculinization of female mice treated with prenatal testosterone (discussed in Level 3).

In an elegant mouse model, called the “Four Core Genotype” (FCG) model, the sex-determining gene *Sry* was manipulated to allow XX embryos to develop into males and XY embryos into females. This mouse model was pioneered by Burgoyne, Lovell-Badge, and colleagues (Burgoyne, 1993; Lovell-Badge & Robertson, 1990; Mahadevaiah et al., 1998) and was generated using two sequential genetic manipulations. First, the *Sry* gene, which is sufficient and necessary to trigger testis development, was deleted in an XY embryo, generating XY^{-} females with ovaries (XYF). Second, a transgenic copy of *Sry* was reintroduced into an XY^{-} embryo (insertion on Chromosome 3) producing an XY^{-}*Sry* mouse that is male and fertile (transgenic XYM). Finally, mating the transgenic XYM mouse with wildtype XX females generates four types of progeny: XX*Sry* males (XXM), XY^{-}*Sry* males (transgenic XYM), XY^{-} females (XYF), and XX females (XXF). Comparing a phenotype of interest in these four animals allows the researcher to attribute sex differences to the gonadal sex factor, or the sex chromosome complement factor in a two-way ANOVA test. We can look for the effects of gonadal secretions in male vs. female comparisons (**XXF vs. XXM**, or **XYF vs. XYM**). We can also test for the effects of sex chromosome complement in XX vs XY

comparisons (**XXF vs. XYF**, or **XXM vs. XYM**). Importantly, the two female groups and two male groups do not show a difference in estradiol and testosterone levels, respectively. These hormone levels have been tested directly in adults (Corre et al., 2016; Gatewood et al., 2006; Manwani et al., 2015; Sasidhar et al., 2012) and indirectly in prenatal mice by measuring the anogenital distance after birth (Itoh et al., 2015). Finally, one could further control for the effects of the *Sry* transgene in XY animals using wild type XY males (**XY^{-}*Sry* vs. XY**).

Two other mouse models are useful in determining which sex chromosome (X or Y) contributes to the sex difference in a given phenotype. The first is the XY* model (Burgoyne et al., 1998; Eicher et al., 1991) where the Y* refers to a variant of the Y chromosome that retained the *Sry* gene but lost the Y-centromere; Y* also gained a duplicated portion of the pseudo-autosomal region (PAR) and a small region of the non-PAR region of the X chromosome (NPX). This duplication allows the Y* chromosome to undergo recombination with the X chromosome during male meiosis to generate Y^{*X} and X^{Y*} chromosomes, respectively. The progeny of a cross between XY* males and wild type XX females are: XY* male, XX female, XY^{*X} (equiv. XO) female and XX^{Y*} (equiv. XXY*) male. [*The Y^{*X} chromosome in the XO mouse is small and comprises almost a normal PAR while the X^{Y*} chromosome in the XXY* mouse is almost a combination of an X and a Y*.*] (Burgoyne & Arnold, 2016). Comparisons of these four genotypes identify the effect of two- vs. one-dose of the X chromosome in males (**XXY* vs XY***), and in females (**XX vs. XO**). We can also discover the effect of presence vs. absence of the Y chromosome in the 1X background (**XO vs. XY***) or the 2X background (**XX vs. XXY***) but gonadal sex may also exert sex-biasing effects here. Importantly, testosterone levels do not differ in animals within gonadal type (Bonthuis et al., 2012). In C57BL/6 XY* mice gonadectomized as adults, XX and XXY* mice gained more weight and fat mass than XO and XY* mice when fed a high-fat diet, suggesting that X chromosome dosage could contribute to sex differences in obesity and metabolism (Chen et al., 2012).

A second useful model to determine the dosage effects of the X and Y chromosome is the Sex Chromosome Trisomy (SCT) model (Chen et al., 2013; Park et al., 2008). Mice in this model are of the outbred MF1 strain due to a lack of robust phenotype in inbred strains. In a cross between an XY^{-}*Sry* male and an XXY^{-} female, the female produces X or XY^{-} bearing ova while the male produces X or Y^{-} bearing sperms with independent segregation of *Sry*. This produces 8 types of progeny: 4 types of females (XX, XY^{-}, XXY^{-}, XY^{-}Y^{-}) and the corresponding *Sry* bearing males (XX*Sry*, XY^{-}*Sry*, XXY^{-}*Sry*, XYY^{-}*Sry*). Within the male or female group, one could identify the effects of an extra X chromosome (**XY^{-} vs. XXY^{-}**), or an extra Y (**XY^{-} vs. XY^{-}Y^{-}**, or **XX vs. XXY^{-}**) when measuring a phenotype of interest. The testosterone levels of these animals do not differ across the genotypes within each sex (Park et al., 2008). Interestingly, gonadectomized and testosterone treated females in the SCT model have 14% higher testosterone concentration in blood than the corresponding males but this difference is not significant if body size is taken into accounted (i.e., total testosterone in blood is similar between males and females).

2.5 Gonadectomy and gonadal hormone treatment in the FCG, XY* and SCT mouse models

Since the levels of gonadal steroid hormones do not differ significantly within sex in these mouse models, the effects of sex chromosome complement (or dosages of X and Y) could technically be studied in these animals without gonadectomy and hormone replacement. However, studies using these mouse models typically include the classic endocrine experiment (gonadectomy and hormone replacement treatment) to physically enforce a uniform level of testosterone or estradiol across all animals at the time of phenotypic assay. A more fundamental reason for performing gonadectomy is to remove the acute effects of gonadal hormones, thereby separating the “activational” and “organizational” effects of gonadal secretions, if any. To test for the “organizational effects” of gonadal secretions, one should further manipulate the testosterone levels in prenatal animals across all genotypes (i.e., increased exposure in female, or reduced exposure in males). However, this “organizational” test is rarely if ever performed in studies using these three mice models.

Section 3: Recent studies using the FCG, XY* and SCT mouse models

A number of studies have used the FCG and/or XY* mouse model to determine the influence of sex chromosome complement on sex-biased traits, such as obesity, food intake, hyperlipidemia, and hypertension (Bonthuis & Rissman, 2013; Chen et al., 2012, 2013, 2015; Link et al., 2015, 2017). A recent, updated list of published papers using these mouse models is reported in (Arnold, 2020). Most of these studies found substantial effects of the sex chromosome complement in addition to the effects of gonadal hormones. Most studies also focused on the assessment of specific tissue functions or organismal-level traits rather than the transcriptomic profile of relevant tissues. Here, I will highlight a few key studies and discuss how they are reshaping our understanding of sex differences.

Study 1: The Number of X Chromosomes Causes Sex Differences in Adiposity in Mice (Chen et al., 2012)

Chen *et al.* first assayed the body weight of FCG mice at postnatal day 21 (P21; age of weaning), P45 and P75 to determine whether sex chromosome complement contributes to sex differences in body weight. At P21, the four groups of FCG mice did not differ in body weight. At P45, gonadal males were 20% heavier than gonadal females (in XX or XY). At P75, gonadal males were 25% (in XX) or 28% (in XY) heavier than corresponding females. With regards to sex chromosome complement, XX mice were heavier than XY mice by 6.3% (in gonadal males) and 8.8% (in gonadal females). Overall, the body weight of gonad-intact adult FCG mice can be described as **M > F** and **XX > XY**.

To resolve the two types of gonadal hormone effects ("activational" or "organizational"), the authors performed gonadectomy (GDX) on FCG mice (at P75) to remove the acute "activational" effects of gonadal hormones. At the time of GDX, the body weight of FCG mice is described as above (**M > F** and **XX > XY**). At 4-weeks post-GDX, the sex difference between males and females disappeared (**M = F**) and body weight of all genotypes converged. At 7-weeks post-GDX, **XX > XY** re-emerged and maintained throughout. At 10-*months* post-GDX, XX mice weighed 24% more than XY mice and strikingly, XX females weighed more than XX males (**F > M**). This reversal of the sex difference in body weight, long after the absence of gonadal hormones, is attributed to the "organizational effect" of gonadal secretions. On top of this, the maintained **XX > XY** difference in body weight, before and after GDX, establishes the contribution of sex chromosome complement to body weight. Overall, the authors reported a significant interaction between the XX complement and "organizational" gonadal hormone effects where "*the male-female difference in X [dosage] acts in opposite directions to the male-female difference in gonadal hormones*".

The body weight difference between the FCG mice (at 10 months post-GDX) is also mirrored in the fat mass, lean mass and plasma leptin differences between the four genotypes. The gain in weight is explained by an almost doubling of total fat mass and a 2- to 3-fold increase in plasma

leptin between XX and XY. Furthermore, XX mice also showed faster weight gain than XY mice when fed a high-fat diet (beginning at 4-weeks post-GDX). XX mice developed fatty liver and showed greater lipid droplet abundance and higher insulin levels than XY mice.

Chen *et al.* also used the XY* mice model to uncover the effects of X dosage vs. Y dosage on sex difference in body weight. All mice underwent GDX at day 75, and their body weight and fat mass were assayed at 9-month post-GDX. Mice with two X chromosomes (XX and XXY) had higher body weight and fat mass than mice with one X chromosome (XY and XO+PAR). Interestingly, the presence of the Y chromosome (XX vs XXY, XO+PAR vs XY) did not show any significant effect on the phenotypes. Finally, the authors found 8 X-inactivation "escapee" genes (*Eif2s3x, Kdm6a, Ddx3x, Kdm5c, Usp9x, Uba1, Rik, Shroom4, Mid1*) that showed higher expression levels in XX than XY mice in the liver, gonadal adipose and/or inguinal adipose tissues. Of these, three genes (*Ddx3x, Uba1, Mid1*) also showed significant differences in expression levels between gonadal female and male mice, suggesting additional gonadal hormone effects on these X-linked genes.

In summary, this was the first study to report the sex chromosome complement effect, independent of gonadal hormones, on sex differences in body weight and fat accumulation. This study closely followed the classic experimental strategy (outline in Section 2) while using the FCG and XY* mouse models. The authors first established the sex difference in gonad-intact animals, and then performed GDX to eliminate the acute "activational" effects of gonadal hormones. They inferred the presence of "organizational" effects when the sex difference persisted long after GDX (lapse of 10-month). A missing piece in this study is the absence of gene expression data for autosomal genes in the various adipose tissues from the FCG mice.

Study 2: The Sex Chromosome Trisomy mouse model of XXY and XYY: metabolism and motor performance (Chen et al., 2013)

The authors used the SCT mouse model, consisting of XXY, XYY, XY, and XX mice with either ovaries or testes, to study the effects of X and Y dosage on the sex difference in weight gain and fat accumulation. In gonad-intact adults, males were significantly heavier (25%) than females (**M>F**), regardless of sex chromosomal complement; XXY mice (regardless of sex) had significantly greater (13%) body weight and relative fat mass (24%) compared to XY mice, and XY did not differ from XX or XYY (**XXY > XY = XX = XYY**). XXY mice also had significantly more (10%) absolute lean mass than XY, but XY did not differ from XX or XYY.

To test for the acute effects of gonadal steroid hormones, gonadectomy (GDX) was performed on all mice in adulthood (at 97-124 days of age) and implanted with a testosterone pellet (T). At 3-weeks post-surgery, testosterone levels in all mice were within the physiological range (170–1,440 ng/dL) with no significant difference between all genotypes. Gonadal males remained significantly heavier (26%) than gonadal females (**M > F**), XXY mice weighed (13%) more than XY mice, and XY animals did not differ from XX or XYY animals (**XXY > XY = XX = XYY**). In terms of fat mass, XXY mice had (43%) more relative fat mass than XY mice, and XX had (21%) more than XY

mice (**XXY > XX > XY**). The difference in weight of key metabolic tissues in these GDX+T mice also mirrored the difference in their body weight. Gonadal and inguinal fat depots, liver, and kidney were significantly heavier in gonadal males than females (**M > F**). In pairwise comparisons, XXY had significantly heavier liver, gonadal fat pad and inguinal fat pad compared to XY, and XY did not differ from XX or XYY (**XXY > XY = XX = XYY**).

With regard to Y dosage effects, no significant differences were found in body weight or metabolic variables between XYY and XY mice, with or without GDX+T (**XYY = XY**). However, XYY males performed worse on the pole test than XY males but no difference was found between the corresponding females (**XYY < XY = XX = XXY; in males**), suggesting that some sex chromosome complement effects might vary between gonadal males and females of the same sex chromosome complement.

In summary, the SCT mice model delineates the effects of X and Y dosage effects on body weight and fat composition, and the XXY- and XYY-biased phenotypes found in mice parallel those found in Klinefelter Syndrome (KS) in humans. In this study, only testosterone was administered after GDX to mimic the hormonal treatment given to KS men. Theoretically, testosterone treatment immediately following GDX is equivalent to gonad-intact males, and this is reflected in the trends in body weight and fat mass (**XXY > XY = XX = XYY**) while comparing gonad-intact and GDX+T groups. However, GDX+T in XX females would not be equivalent to gonad-intact XX females since normal females have lower testosterone levels than males. For future studies, one might be interested to study the effects of testosterone treatment in GDX females or estradiol treatment in GDX males to uncover other latent sex differences.

Study 3: Diet, gonadal sex, and sex chromosome complement influence white adipose tissue miRNA expression (Link et al., 2017)

The FCG mouse model was used to assess the miRNA expression profile in the gonadal fat tissue under three physiological conditions – normal condition (chow-diet, gonad-intact), high-fat diet, and gonadectomy (GDX). In the normal condition, the distribution of female-to-male miRNA read count ratios was male-biased (**XXM+XYM > XXF+XYF**) and no difference was observed between XX vs. XY groups (**XX = XY**). When gonadectomy was performed in adult FCG mice (at 10 weeks of age) and their gonadal fat depots assessed for miRNA expression at 5-months post-GDX, a sex-reversal in the distribution of female-male miRNA read count ratios was observed (**XXM+XYM < XXF+XYF**) and XX vs. XY comparisons showed no difference (**XX = XY**). These results suggest that gonadal secretions have an acute "activational" effect on the miRNA expression profile.

To study the effect of high-fat diet on the miRNA expression profile, the authors performed GDX in chow-fed adult FCG mice (10 weeks of age) and at 1-month post-GDX, switched to a high-fat diet for 4 months. Comparing GDX animals on high-fat diet, a significant male-bias in the distribution of female-to-male miRNA read count ratios was found (**XXM+XYM > XXF+XYF**). In addition, the high fat diet induced an XY-bias in the distribution of miRNA read count ratios (**XY >**

XX). Principle component analysis of the miRNA expressions in all samples showed that diet accounted for the greatest variation in miRNA expressions (chow-diet vs. high fat-diet groups separated in PC1; 40.1% explained variance), followed by gonadal state (gonad-intact vs. GDX in PC2; 16.2%) and gonadal type (testes vs. ovaries in PC3; 12.4%). Sex chromosome complement (XX vs. XY) showed little divergence in all principal components. Performing the PCA analysis in chow-diet samples only, gonadal status was separable in PC1 (35%) and PC2 (20%), gonadal type clustered separately in PC3 (18.8%) and PC4 (10.4%), and XX and XY groups showed slight separation in PC3 and PC4.

Overall, gonadal steroid hormones but not sex chromosome complement contribute to the sex difference in miRNA expressions in gonad-intact FCG mice. More interestingly, in GDX animals, a high-fat diet induced sex differences dependent on sex chromosome complement, and reversed the sex differences (in the chow-diet group) in miRNA expression. This suggests that diet could induce sex-biased regulation of miRNA expression levels in the mouse.

Systems approach in the study of sex differences

From these representative studies, we see that the FCG model is a suitable model for determining the effects of gonadal steroid hormones ("organizational" and "activational") and sex chromosome complement. However, testing whether each of these sex-biasing factors contributes to a sex difference is only a first step towards understanding the mechanisms underlying that sex difference. Each sex-biased phenotype is a manifestation of many sex-biases in molecular factors found in tissues/cells (Godfrey et al., 2020; Naqvi et al., 2019), and we should study these sex-biased molecular factors using a systems approach, i.e., to identify all relevant molecular factors that underlie a sex difference in phenotype (e.g., gene expression profiles) and probe each molecular factor's effect on the rest of the system, including the phenotypic outcome. Uncovering interactions among sex-biased molecular factors will ultimately help us to explain some sex differences at the mechanistic level and explore sex differences in terms of gene regulatory networks.

Concluding Remarks

The two classic sets of sex-biasing factors (sex hormones and sex chromosomes) arise early in embryonic development and trigger numerous downstream sex differentiation events, which in turn influence numerous sex differences over a lifetime. The sex hormones and sex chromosomes are therefore part of a larger collection of sex-biased factors. A systematic catalog of genetic effects (e.g., changes in gene regulatory networks) of these factors will bring us closer to understanding the *sexome* – "the sum of sex-biased effects on gene networks and cell system" (Arnold & Lusis, 2012). Ultimately, we will rely on high-throughput approaches to identify new sex-biased molecular components and large-scale perturbation assays that demonstrate interactions between these components to probe the mechanisms underlying sex differences in humans and animals.

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