Published in final edited form as:

Horm Behav. 2012 April ; 61(4): 565–572. doi:10.1016/j.yhbeh.2012.02.003.

X-chromosome dosage affects male sexual behavior

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Abstract

Sex differences in the brain and behavior are primarily attributed to dichotomous androgen exposure between males and females during neonatal development, as well as adult responses to gonadal hormones. Here we tested an alternative hypothesis and asked if sex chromosome complement influences male copulatory behavior, a standard behavior for studies of sexual differentiation. We used two mouse models with non-canonical associations between chromosomal and gonadal sex. In both models, we found evidence for sex chromosome complement as an important factor regulating sex differences in the expression of masculine sexual behavior. Counter intuitively, males with two X-chromosomes were faster to ejaculate and display more ejaculations than males with a single X. Moreover, mice of both sexes with two X-chromosomes displayed increased frequencies of mounts and thrusts. We speculate that expression levels of a yet to be discovered gene(s) on the X-chromosome may affect sexual behavior in mice and perhaps in other mammals.

Keywords

Sexual differentiation; Klinefelter's; Turner Syndrome; Aneuploidy; sex difference; Xinactivation

Introduction

The original studies on mammalian sexual differentiation of behavior identified androgen, produced in the developing testes, as the critical factor responsible for differences between adult male and female behavior (Phoenix et al., 1959). Testosterone, acting both directly, and after aromatization to estradiol, binds to its receptors in the brain and modifies neural circuits. In adults, these modifications lead to increased expression of behaviors more typical of males, and decreased display of behaviors more often shown by females. However, in addition to androgen differences, there are genetic differences between males and females caused by unequal dosage of sex chromosome genes. In mammals, and many other species, sex is determined by genetic inheritance of sex chromosomes. Normal female mammals (XX) have two X-chromosomes while males (XY) have a single X- and a Y-chromosome. The X-chromosome encodes hundreds of genes with no direct homologues on Y, whereas the Y-chromosome encodes many fewer genes, including the testis-determining factor *Sry* (Ellegren, 2011; Koopman et al., 1991). In addition to *Sry*, genetic differences

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between XX and XY individuals are now recognized as a source of variation that shapes sex differences in brain and behaviors (Arnold, 2009).

Over the last decade, several mutant mice with atypical sex chromosome arrangements have been developed and used to test the effects of sex chromosome complement on sexual differentiation (Arnold, 2009). Here we employ the Four Core Genotypes (FCG) and Y* models, which are described in Table 1. In FCG mice, males and females can have either XX or XY sex chromosomes (De Vries et al., 2002). *Sry* (testis determining gene) is deleted on the FCG Y-chromosome and a transgenic copy of *Sry* is located on an autosome, thereby unlinking differentiation of the gonads from the sex chromosomes. Autosomal inheritance of the *Sry* transgene causes testes development in both XX and XY mice (gonadal males), and ovaries develop in mice without the autosomal transgene (gonadal females). Since same gonadal sex FCG mice differ by XX and XY genotypes, sex chromosome effects revealed by the FCG can be attributed to one of three major mechanisms: genes on Y, genes that escape X-inactivation, and paternally imprinted X-genes.

The Y* model is used to determine whether sex chromosome effects present in the FCG are due to dose of X- or Y-chromosome genes. The Y*-chromosome was generated by a spontaneous translocation and inverted duplication of the pseudoautosomal region (PAR) of Y (Eicher et al., 1991). During meiosis, the altered PAR of Y* recombines aberrantly with the X-chromosome and generates male gametes with four sex chromosomes: non-recombined X and Y*, and recombined Y*X (PAR without unique X and Y genes) and XY* (an X attached to Y chromosome). Gonadal male and female Y* mice can have one or two copies of the X-chromosome, whereas only males have a Y-chromosome. Therefore, while FCG XX and XY genotypes differ in both dose of X and presence of Y, Y* mice of the same gonadal sex only differ in dose of X (Table 1). If dosage of X-chromosome genes is important for the observed differences in FCG, we expect to see the same, or perhaps more pronounced, sex chromosome effects in Y* mice.

Using these mouse models, herein we examined the sex chromosome hypothesis by studying two highly sexually dimorphic behaviors: masculine sexual behavior and aggression (Bonthuis et al., 2010). We found a strong effect of X-chromosome number on several aspects of masculine sexual behavior; counter intuitively individuals with two X-chromosomes displayed more behavior than mice with one X-chromosome. The X-chromosome effect did not generalize to sexually dimorphic resident-intruder aggression, or dimorphic vasopressin (AVP) density in the lateral septum of Y* mice. Our data indicate that two X-chromosomes increase male sexual behavior in mice, but the Y-chromosome may increase aggression and AVP immunoreactivity in the lateral septum.

Methods

Animals

All animal care and procedures were performed in accordance with and approved by the University of Virginia Animal Care and Use Committee. Adult (55–75 days old) FCG and Y* mice in the C57BL/6J background strain (see Table 1) were gonadectomized and implanted (SC) with Silastic tubing (1.02 mm inner diameter × 2.16 mm outer diameter; Dow Corning) filled with 1cm of crystalline testosterone while under general isoflurane anesthesia. The mice were housed alone on a 12hr reverse light cycle with 2400 light onset and 1200 dark onset (EST), and given food (diet # 7912; Harlan Teklad, Indianapolis, IN) and water *ad libitum*. In Experiment 1, 12 mice per genotype (total n=48) were used for FCG male sex behavior tests. Our Y* breeding colony was originally started with B6Ei.LT-Y*/EiJ males and C57BL/6J EiJ females purchased from The Jackson Laboratory (Bar Harbor, ME; stock numbers 002021 and 000924). The mice in our lab were maintained in the B6Ei

substrain. For Y* male sex behavior tests we used n=8 XY*X, n=12 XX, n=18 XY*, and n=16 XX^{Y*} mice. In Experiment 3 (aggression) we used n=14 XY*X, n=14 XX, n=20 XY*, and n=20 XX^{Y*} mice. FCG mice were genotyped by PCR of the YMT2/B-related *Ssty* family on the Y-chromosome (Gatewood et al., 2006), and Y* mice by amplification of reverse transcription of RNA and PCR amplification of *Xist* (Park et al., 2008) as previously described.

Anogenital Distance

Anogenital distance (AGD) was measured in a separate cohort of 96 Y* mice (n = 20 1XM, 24 2XM, 24 1XF, and 28 2XF) on postnatal day 10. Using dial calipers with 0.1mm precision, the shortest distance was measured between the ventral perimeter of the anus to the dorsal perimeter of the genitalia.

Testosterone Radioimmunoassay

Immediately before gonadectomy, blood was collected from each anesthetized mouse by a suborbital puncture, spun and serum frozen. After behavioral testing, serum was collected from trunk blood at time of euthanasia. Testosterone measurements were performed in singlet reactions using Diagnostics Products Corporation testosterone RIA with a detectable range of 0.138–7.487 ng/ml. University of Virginia Ligand Core laboratory performed the assays (supported by NICHD (SCCPRR) Grant U54-HD28934).

Male Sex Behavior

Male sex behavior was tested once a week for four consecutive weeks with hormone primed, sexually experienced, ovariectomized, stimulus C57BL/6J females as previously described (Park et al., 2009). Trials were video recorded in the dark under red-light illumination between 1200–1800h EST. An observer blind to genotypes scored the following behaviors: time to first mount, time to first intromission, number of mounts, number of deep thrusts in each mount, and time to ejaculation.

Resident-Intruder Aggression

One to two weeks after surgery, mice were given three days of social exposure (Sipos and Nyby, 1998). Beginning two days later, each mouse was tested on three consecutive days for resident-intruder aggression. Tests were conducted in the dark under red-light illumination between 1200–1700h (Gatewood et al., 2006). The intruders were anosmic C57BL/6J males. Anosmia was induced with Dichlobenil (2,6 Dichlorobenzonitrile) and verified by a hidden cookie test (Brandt et al., 1990). Tests were 10 minutes in duration or ended when the resident first attacked the intruder, and attack latencies were scored.

Tissue Preparation and Vasopressin Immunocytochemistry

Y* mice from the previous behavioral experiments were deeply anesthetized with isoflurane inhalant, and brains were extracted and fixed by submersion in 4% acrolein in PBS for 4hrs. Brains were removed, placed in 30% sucrose overnight, and frozen on dry ice. Tissue was cut into 30 μ m coronal sections, collected into four vials, and then stored in antifreeze (30% w/v sucrose, 1% w/v PVP 40, 30% v/v ethylene glycol in 0.02M TBS) at -20°C until processing. Immunocytochemical staining for vasopressin was performed as previously described (Scordalakes and Rissman, 2004). The density of vasopressin immunoreactive fibers (AVP-IR) in the lateral septum was determined using microscopy and MetaMorph (Molecular Devices, Sunnydale, CA) image analysis software of a standard area that surrounded the region. The defined region for the rostral lateral septum was a rectangle of about $4.6 \times 10^5 \text{ um}^2$, while the caudal septum was defined by a triangle of approximately $5.2 \times 10^5 \text{ um}^2$. The regions were aligned on the images with the edge just medial to the

lateral ventricles. A researcher blind to sex and genotype quantified both left and right sides of one rostral section corresponding to figure 27, and one caudal section corresponding to figure 29, in the mouse brain atlas of Paxinos and Franklin (Franklin and Paxinos, 2008), in a similar manner as described (Rood et al., 2008). The average area stained was then calculated.

Quantitative Real-Time PCR

Y* mice were anesthetized as above and brains were frozen on crushed dry ice. Fresh frozen tissue was cut into 120 μ M coronal sections in a cryostat, frozen on glass microscope slides, and RNA was isolated from punches of preoptic area of the hypothalamus (POA) using RNeasy spin column purification (Qiagen, Valencia, CA) as described (Park et al., 2009). cDNA was made from 100ng of RNA using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA), and qRT-PCR was performed in triplicate reactions with Fast SYBR Green Master Mix (Applied Biosystems), 1ng cDNA, and 100nM primers reactions on an ABI StepOnePlus thermal cycler (Applied Biosystems). No reverse transcriptase control reactions ruled out amplification from genomic DNA. Oligonucleotide primers (Invitrogen) were designed as previously described (Park et al., 2009) for the androgen receptor, aromatase, estrogen receptor- α , and cyclophilin B (endogenous control) mRNA transcripts (Table 2). All primer-pairs were verified to be 90–110% efficient in standard curve reactions and amplified a single product determined by melting-curve analysis. Relative quantifications of mRNA levels were measured by the $\Delta\Delta$ Ct method with StepOne Software v2.2.

Statistics

Median latencies to first mounts, thrusts (interval between first mount and first thrust), ejaculations (interval between first thrust and ejaculation), and attacks were determined with Kaplan-Meier curve analyses using the sum of time over all four trials (4 trials $\times$ 60 min. per trial = 240 min. total), and statistical differences between curves were measured with Log-rank (Mantel-Cox) Tests in GraphPad Prism 5 software. Differences in number of mice mounting and thrusting in a criterion number of trials were measured by Fisher's Exact tests, and relative risks (RR) with their 95% confidence intervals (ci) are reported. Chi-squares tests (corrected for a small n) were used for differences in the number of trials in which males ejaculated. The average number of mounts per trial, thrusts per trial, thrusts per mount per trial (averages only included mice that displayed the behaviors; Fig. 2), mounts per minute per trial, thrusts per minute per trial (counted after initial mount and thrust, respectively, within a trial; Fig. 3), testosterone levels, anogenital distance, and AVP area were measured by two-way ANOVA using gonadal sex and X-chromosome dosage as the two factors. Planned comparisons were conducted with Fisher LSD tests. ANOVA effect sizes are reported with eta-squared (η^2). Pearson product moment was used to test for correlations of testosterone concentration and male sex behaviors.

Results

Sex chromosome complement affects masculine sexual behavior

Males in the FCG with two X-chromosomes (XXM) were faster to ejaculate than males with a single X- and a Y- (XYM) chromosome (Fig. 1a; $\chi^2_1=7.68$, $P<0.01$). The median ejaculation latency of XXM at 14.2 minutes was less than half the median latency of XYM at 37.6 minutes. XX males also ejaculated in more trials than XYM, but this difference was not statistically significant (Fig. 1b). Additionally, mice with an XX genotype (both XXF and XXM) generally engaged in mounts and thrusts in more trials than XY mice, and XXF performed them more frequently (Fig. 2) and at a faster rate (Fig. 3) during individual trials than XYF, but differences were not significant.

Individuals with two X-chromosomes display more masculine sexual behavior

In order to determine whether the source of the significant difference in ejaculatory behaviors between FCG males was due to the presence of the Y-chromosome, or the presence of a second X-chromosome of paternal origin (See Table 1), we tested male sexual behavior in Y* mice. While it took some males with a single X-chromosome (1XM) a relatively long time, over the duration of four trials, to ejaculate their median ejaculation latency (1XM = 35.5 min.) was not different than males with two X-chromosomes (2XM = 35.2 min; Fig. 1c). Strikingly, a significantly greater fraction of 2XM than 1XM ejaculated in one or multiple trials (Fig. 1d; $\chi^2_2=9.46$, $P<0.01$). Therefore, possession of two X-chromosomes increased ejaculation frequency.

For nearly all measures of mounting and thrusting examined within Y* mice of the same sex, large differences in behavior could be attributed to X-gene dose. Females with two X-chromosomes (2XF) were faster to initiate mounting than one X (1XF) females (median latency, 2XF 4.74 min., 1XF 7.36 min; $\chi^2_1=7.28$, $P<0.01$). In addition, all 2XF (12 of 12) mounted and thrust in all four trials compared to only half (4 of 8) of the 1XF (RR=2.00, 1.00–4.00ci; $P<0.05$). Similarly, nearly all 2XM (14 of 16) compared to only half of the 1XM (9 of 18), mounted in a majority of the trials (3–4 trials), and all 2XM thrust during at least one trial, whereas five of the 1XM failed to thrust in any trial (mounts RR=1.75, 1.06–2.88ci; thrusts RR=1.39, 1.04–1.84ci; $P<0.05$ for both comparisons).

Significant effects of X-chromosome dosage, and interactions between sex and X dose, were noted for the number of mounts ($F_{1,48}=10.7$, 4.22; $\eta^2=0.162$, 0.064; $P<0.01$, $P<0.05$ respectively) and thrusts ($F_{1,43}=9.23$, 4.78; $\eta^2=0.161$, 0.083; $P<0.01$, $P<0.05$ respectively, Fig. 2) per trial. 2XF displayed significantly more mounts and thrusts than all other groups (Fig. 2d,e). In addition, the average number of thrusts per mount, ($F_{1,43}=8.61$; $\eta^2=0.165$; $P<0.01$), and mounts and thrusts per minute ($F_{1,48}=12.3$, $F_{1,42}=7.48$; $\eta^2=0.191$, 0.078; $P<0.01$ or less), were all affected by the dose of X and in each case 2X mice displayed more behavior than 1X mice (Fig. 2f, and Fig. 3c,d). Importantly, the high levels of mounting and thrusting noted for 2XM were not correlated with ability to ejaculate since 2XM were just as fast, and in some cases faster, to attain the first ejaculation as 1XM (Fig. 1c), and there were no differences in the median number of mounts and thrusts per ejaculation between the two groups (data not shown).

Females display high frequencies of masculine sexual behaviors

FCG females treated with testosterone in adulthood were faster to initiate and engaged more frequently in mounting and thrusting than males. Nine of 12 XXF thrust in all four trials compared to 25% of the XXM (RR=3.00, 1.07–8.43ci; $P<0.05$), two-thirds of the XYF and only 2 of 12 XYM displayed thrusts in all four trials (RR=4, 1.06–15.1ci; $P<0.05$). Gonadal sex differences were also found in numbers of mounts ($F_{1,38}=26.7$, $\eta^2=0.395$; $P<0.0001$), thrusts ($F_{1,37}=36.20$, $\eta^2=0.478$; $P<0.0001$), and thrusts/mount ($F_{1,37}=5.95$, $\eta^2=0.133$; $P<0.05$) per trial (Fig. 2a–c). In all cases, FCG females exhibited more behavior than males. The numbers of mounts/min. and thrusts/min. during sex behavior were calculated after the initial mount and the initial thrust in a trial, respectively. There was an effect of sex on thrusts/min ($F_{1,37}=14.0$, $\eta^2=0.140$; $P<0.05$); and, in this case the average per trial was higher in FCG males (Fig. 3b).

Like the FCG, Y* females were generally faster to initiate and more frequently engaged in mounting and thrusting than males. All 2XF mice mounted in all trials compared to only 10 of 16 2XM (RR=1.60, 1.10–2.34ci; $P<0.05$). 2X females were faster to thrust than 2XM (median latency, 2XF 0.17 min. < 2XM 4.53 min; $\chi^2_1=9.12$, $P<0.01$), and all 2XF thrust in all trials compared to 4 of 16 2XM (RR=4, 1.71–9.35ci; $P<0.0001$). Also like the FCG

mice, thrusts/min. was the only measure in which Y* males were significantly higher than females (Fig. 3d; $F_{1,42}=45.3$, $\eta^2=0.490$; $P<0.0001$).

Males displayed aggression toward an intruder, and had more dense vasopressin immunoreactivity than females

Unlike males, females never attacked in the resident-intruder task, thus demonstrating a large gonadal sex difference in aggression. There were trends for 2X males to attack intruders faster ($\chi^2_1=2.80$, $P<0.1$), and attack in more trials ($\chi^2_2=5.33$, $P<0.07$), than 1XM (Fig. 4). Therefore, aggression in the Y* model is clearly affected by gonadal sex or presence of the Y-chromosome, while X-chromosome number may play only a minor role.

In the same Y* mice used for aggression, density of vasopressin (AVP) immunoreactive (ir) fibers in the lateral septum was sexually dimorphic with males having more dense AVP-ir than females ($F_{1,32}=4.98$, $\eta^2=0.134$; $P<0.05$). We found no effect of X-chromosome dosage on fiber density (Fig. 5). This finding indicates that X dosage in the brain does not directly affect the AVP-ir, and X dosage does not indirectly alter the AVP-ir through changes in steroid hormone levels.

X-chromosome copy number does not affect anogenital distances, serum testosterone, or expression of androgen receptor, aromatase, or estrogen receptor- α

In a separate cohort of animals, anogenital distances (AGD) were measured on postnatal day 10; this reflects androgen levels during gestation (Gandelman et al., 1979; vom Saal and Bronson, 1980). In the Y* mice, large gonadal sex differences ($M>F$; $F_{1,92}=702$, $\eta^2=0.881$; $P<0.0001$), but no effect of X-chromosome dosage ($F_{1,92}=0.97$, $\eta^2=0.001$) were found on post natal day 10 (Mean $\pm$ SEM in mm; 1XM = 3.68 ± 0.08 , $n=20$; 2XM = 3.66 ± 0.10 , $n=24$; 1XF = 1.73 ± 0.05 , $n=24$; 2XF = 1.90 ± 0.05 , $n=28$). These data indicate that X-chromosome dosage does not influence androgen exposure in utero.

Gonad-intact adult Y* females had lower serum testosterone levels than males, but only a trend was noted ($F_{1,73}=3.01$, $\eta^2=0.039$; $P=0.08$). Importantly, testosterone levels were unaffected by number of X-chromosomes in both intact males and females ($F_{1,73}=1.26$, $\eta^2=0.016$, Table 3).

Differences in serum testosterone were not detected between adult gonadectomized 1X and 2X (male and female) mice replaced with exogenous hormone ($F_{1,56}=1.53$; $\eta^2=0.026$, Table 3). Females did have slightly higher serum levels than males ($F_{1,57}=6.78$, $\eta^2=0.101$; $P<0.05$); however, since the same testosterone dose was used for all animals, and females are smaller than males, accounting for body-weight as a covariate eliminated the gonadal sex difference ($F_{1,56}=2.19$, $\eta^2=0.036$; $P=0.15$). Moreover, the small variability in serum testosterone of Y* males ($n=12$ 1XM, $n=10$ 2XM) did not correlate with the number of mounts, thrusts, thrusts per mount, mounts per minute, and thrusts per minute per trial ($R^2<0.014$ for all measures). When genotypes were measured separately, only 2XM came close to a negative correlation trend between the single measure of thrusts per mount and testosterone concentration ($R=-0.599$, $(-)0.892-(+)0.048$ ci; $P=0.07$). This is the reverse of what might be expected, that higher T would be correlated with more sexual behavior, therefore, the X-chromosome dosage effects on male sex behavior do not appear to be confounded by differences in activational testosterone levels.

To assess whether X-chromosome dosage could alter expression of androgen receptor, estrogen receptor α , or aromatase, qRT-PCR was used to measure mRNA transcript levels of *Ar*, *Esr1*, and *Cyp19a1* in the preoptic area of the hypothalamus (POA). No significant differences were found in expression levels of any of these genes (Fig. 6).

Discussion

Using two mouse models that allow separation of the effects of gonadal determination and dichotomous androgen exposure from direct genetic effects of sex chromosomes, we noted that male mice with two X-chromosomes ejaculated more frequently than males with a single X-chromosome. The observations that FCG XXM had an ejaculation frequency similar to Y* 2XM, and XXM were significantly faster to ejaculate than XYM, provided evidence that the X-chromosome dosage effects in Y* mice are not a consequence of sex chromosome aneuploidy (XXY). Measurements of mounting and thrusting frequency, in both Y* males and females, further supported the enhancement of male sexual behavior by a dose of two X-chromosomes.

It is important to note that these experiments were conducted with gonadectomized mice treated with T-implants, which restored and normalized T levels to those present in normal gonad-intact C57BL/6J adult males. Neonatal AGD measurements indicated that perinatal androgen levels were identical between mice of the same gonadal sex. Similarly, no peripubertal hormone differences have been detected in either the FCG (Cox and Rissman, 2011) or the Y* mice (Cox and Rissman, unpublished data) at postnatal days 21 and 30. Therefore, it is not likely that androgen secretions are grossly altered by sex chromosome complement in our mouse models during development. Adult testosterone measurements were also unaffected by sex chromosome complement, as previously shown for both Y* (Wistuba et al., 2010) and FCG mice (Gatewood et al., 2006; Palaszynski et al., 2005). Our results show that sex chromosome copy number, separate from the classical mechanism of dichotomous perinatal androgen exposure from the gonads, influences male sex behavior.

In addition, we found no differences in the expression of genes encoding the androgen receptor, aromatase enzyme, or estrogen receptor- α in the POA of adult mice, a hormone sensitive brain region essential for male sex behavior (Hull and Dominguez, 2007). Although the above observations cannot preclude small perturbations in androgen secretions during development, and differences in steroid hormone metabolism and signaling, to date there is no evidence that sex chromosome complement alters these steroidal mechanisms in the FCG and Y* models. Indeed, the higher levels of mounting and thrusting in 2XF as compared to 1XF also document that the sex chromosome complement effects on male sex behavior are in fact due to X-chromosome dosage, not perinatal androgen exposure.

In our first publication on behavior in the FCG mice we did not report any sex chromosome complement effects on male sex behavior (De Vries et al., 2002), but, unlike our current findings in C57BL/6J FCG, the FCG mice used in that study were in the random bred MF1 background strain. It is known that the genetic background can cause differences within a genetic model (Dominguez-Salazar et al., 2004). Another study on sex chromosome aneuploidy, again in MF1 mice, also did not reveal X-chromosome effects on male sex behavior (Park et al., 2008). However, in the aneuploid experiments, it was not possible to make direct comparisons between mice that differed only in copies of X, and comparisons were further confounded by differences in numbers of both transgenic and endogenous copies of *Sry*, and copies of Y from two genetic background strains.

In light of the findings in Y* mice, it is somewhat surprising that sex chromosome complement effects were not as prevalent in FCG as in the Y* mice. The Y* and FCG mice presently reported are in the same C57BL/6J background strain, but there are genetic differences between the two lines. First of all, Y* mice have been bred separately into their own C57BL/6J sub-strain (Eicher et al., 1991). Secondly, the autosomally inserted *Sry* transgene in the FCG mice could have different expression patterns in the brain than the endogenous *Sry* located on the Y-chromosome. Furthermore, the *Sry*-deleted Y-chromosome

in FCG mice originates from the SV/129 background strain (De Vries et al., 2002), while the Y* chromosome evolved from the Y of C57BL/6J. It has been shown, in mice, that the strain origin of the Y-chromosome can affect sexual mounting behaviors (Shrenker and Maxson, 1983, 1984). Most importantly, unlike the Y* model, the FCG cannot differentiate sex chromosome effects resulting from dose of X, from effects resulting from the presence or absence of Y. Every XX FCG mouse lacks a Y, and every FCG mouse with only one X also has a Y (Table 1). In fact, considering the differences in the two models, it is possible that the short median ejaculation latency of about 15 minutes in XXM, is due to the lack of expression of a Y-chromosome gene that is present in XYM, 1XM, and 2XM that all have median latencies just over 35 minutes. Even though X-chromosome dosage effects are apparent for Y* mounting, thrusting, and ejaculation frequencies, it is conceivable that FCG Y-genes are able to mask X-chromosome dosage effects in FCG mice.

Importantly, while male sexual behaviors are influenced by X-chromosome gene dose, a second X-chromosome does not enhance all male-typical behaviors. In Y* mice, testosterone implanted, ovariectomized females of both genotypes never attacked an intruder male, and there were no differences in number or latencies to attack between males of the two genotypes. As expected, perinatal androgens appeared to masculinize aggression. Our lab previously reported that ovariectomized, testosterone implanted XYF FCG mice in the C57BL/6J background were faster to attack than XXF, and no different from males, in a similar resident-intruder test (Gatewood et al., 2006). Therefore, the absence of Y-genes and aggressive behavior in both 1XF and 2XF, in contrast to high levels of aggression in XYF, can be interpreted as Y-genes increasing aggression in XYF. In fact, it may be that XYF are aggressive because they specifically carry the 129-Y chromosome. In testing conditions similar to the ones used in our lab, 129 males attack an intruder more times during a test than C57BL/6 males (Abramov et al., 2008), and gene(s) on the Y-chromosome can contribute to strain differences in inter-male aggression (Maxson et al., 1979; Shrenker and Maxson, 1983). Moreover, we also found more dense AVP immunoreactivity in the lateral septum of Y* males than in females. These data, combined with previous discoveries of higher levels of vasopressin in XY FCG mice of both sexes (De Vries et al., 2002; Gatewood et al., 2006; Pierman et al., 2008), indicate that Y-chromosome genes also contribute to vasopressin fiber density in the lateral septum. Vasopressin influences aggression in rodents (Albers, 2011; Bester-Meredith et al., 1999; Compaa et al., 1993; Wersinger et al., 2002), and these findings are consistent with a causal link between a gene(s) on the Y-chromosome, vasopressin, and sex differences in aggression. Moreover, it does not appear that 2XF brains were generally more masculinized by perinatal androgens than 1XF, because aggression and vasopressin immunoreactivity were the same and feminine in both genotypes.

A perplexing question from the current findings is: why should a female-typical sex chromosome complement increase male sexual behavior? One possible explanation may be that X-chromosome genes provide “compensation” for the unequal androgen experiences between genetically normal males and females (De Vries, 2004). Relevant to this hypothesis, testosterone treated 2XF show mounting and thrusting levels that are always either higher than 1XM, or in the case of thrusts/min., at least closer to 1XM than are 1XF. These results are consistent with data collected from several strains of genetically homogenous inbred mice showing high levels of mounting and thrusting in testosterone treated females (De Vries et al., 2002; Wersinger et al., 1997). The ability of females to display mounting and thrusting behaviors with shorter latency and at higher levels than males is in conflict with the classical view of sexual differentiation that androgens during early development masculinize and defeminize sexually dimorphic behaviors. On the other hand, the ejaculatory reflex is completely absent in genetic female mice, and perhaps females display more mounting and thrusting simply because they are not capable of

intromission and ejaculation. Alternatively, mounting and thrusting, per se, in female rodents may be unrelated to sexual behavior and could serve purposes of social dominance among females living in groups (Fang and Clemens, 1999), and/or promoting weaning of offspring (Curley et al., 2009). Then again, our females were implanted with testosterone capsules and the mounting and thrusting displayed may not be physiologically relevant to normal female social behaviors.

Mechanistically, X-chromosome dosage effects can be the result of paternally imprinted X-genes, genes that escape X-inactivation, or cell-to-cell X-chromosome mosaics (Arnold, 2004; Berletch et al., 2011; Carrel et al., 1999). About 15% of X-specific genes are transcribed from both the active and inactive X-chromosome in humans (Carrel and Willard, 2005), and about 3% also escape in mice (Berletch et al., 2010; Yang et al., 2010). Genes that escape X-inactivation are more highly expressed in brains, and other tissues, with two X-chromosomes (Werler et al., 2011; Xu et al., 2008a; Xu et al., 2008b). Sex chromosome complement and X-gene dosage have also been shown to affect autosomal gene and protein expression in the brain. For instance, studies using the same models we describe here show that mice with two X-chromosomes have higher calbindin mRNA expression in the cerebellum and frontal cortex (Abel et al., 2011), and higher prodynorphin mRNA expression in the striatum (Chen et al., 2009).

Lessons learned from these mice may be relevant to human aneuploid conditions. It is estimated (based on prenatal sampling) that up to 1 in 600 men have Klinefelter's Syndrome (47,XXY) (Visootsak et al., 2001). Investigations with Y* mice indicate that 2XM recapitulate some phenotypes associated with the syndrome, including prepubertal germ cell loss, Leydig cell hyperplasia (Wistuba et al., 2010), and memory impairments (Lewejohann et al., 2009). But different from many Klinefelter's patients, during puberty and early adulthood, 2XM and 1XM mice have equivalent testosterone levels (Wistuba et al., 2010). Nevertheless, this fact allows us to use the mouse as a model organism for studying symptoms caused directly by X-chromosome aneuploidy, independent of lower androgen levels. The observation that 2XM mice display enhanced male sexual behavior could also translate to the human condition. In a study of subfertile men, Klinefelter's patients reported having sexual intercourse more frequently than 46,XY control males (Yoshida et al., 1997). Furthermore, Klinefelter's patients seeking treatment for sexual dysfunction did not differ from testosterone-matched control men in regard to hypoactive sexual desire (Corona et al., 2010). In normal men sexual activity does not correlate with testosterone levels (Park and Rissman, 2007). Thus, it is possible that some differences in sexual activity in men may be caused by variation in expression of a yet to be identified gene on the X-chromosome.

Acknowledgments

We thank Michelle Edwards, James Patteson, Henry Chan, Ann Kwon, Savera Shetty, and Aileen Ryalls for excellent technical assistance. We thank Dr. Mark Conaway for advice on statistical tests. This work was supported by NIH NS55218. PJB was supported by NIH Training Grant T32 GM08715. KHC was supported by NIH Training Grant T32 HD007323. Testosterone measurements were performed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core NICHD (SCCPRR) Grant U54-HD28934.

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Highlights

Males with two X-chromosomes ejaculate more often than males with a single X-chromosome.

Male mice with two X-chromosomes are faster to attain ejaculations than males with one X-chromosome.

In both sexes, an additional X-chromosome facilitates display of mounting and thrusting.

Fig. 1.

Male mice with two X-chromosomes have enhanced ejaculation capacities compared to males with a single X-chromosome. All mice were castrated and treated with testosterone as adults (Cast+T). **(a & c)** Kaplan-Meier curves for the percent accumulation of males reaching their first ejaculation over time in minutes (defined as the interval between the 1st thrust and the first ejaculation) during all four, one-hour trials. **(b & d)** Histograms of the number of mice ejaculating during 0 (white bars), 1 (lined bars), or 2+ (two or more; black bars) trials. **(a)** Males in the Four Core Genotypes (FCG) with two X-chromosomes (XXM; solid line) were significantly faster to ejaculate than males with a single X-chromosome (XYM, dashed line; **, $P < 0.01$). **(b)** XXM ejaculated in more trials than XYM, but this difference was not significant. **(c)** Latencies to the first ejaculation were not different between Y* males with two copies of X (2XM, solid line) or one copy of X (1XM, dashed line). **(d)** 2XM ejaculated in more trials than 1XM (**, $P < 0.01$). XXM n=12. XYM n=12. 2XM n=16. 1XM n=18.

Fig. 2.

Y* mice with two X-chromosomes mount and thrust more frequently during individual trials than mice with one X-chromosome. Mean $\pm$ SEM for mounting and thrusting behaviors in adult testosterone treated ovariectomized females (OVX+T) and castrated males (Cast+T) tested with receptive females; only mice that displayed the behaviors at least once were included in the calculations. (a–c) Histograms of Four Core Genotypes (FCG) behaviors. XX, XX genotype (black bars). XY, XY genotype (white bars). (d–f) Histograms of Y* behaviors. 2X, two X-chromosomes genotype (black bars). 1X, one X-chromosome genotype (white bars). FCG females have higher numbers of (a) mounts, (b) thrusts, and (c) thrusts per mount per trial than males. Y* females with two X-chromosome (2XF) have higher numbers of (d) mounts, and (e) thrusts per trial than all other groups. (f) 2X Y* mice have a higher number of thrusts per mount per trial than 1X. *Significantly different than all

other groups ($P<0.05$). **Significant difference between mice with two X-chromosomes versus one ($P<0.05$). #Significant gonadal sex difference ($P<0.05$). Panels a–c: XX females (XXF) $n=11$, XX males (XXM) $n=11$, XYM $n=9$. Panel a: XYF $n=11$. Panels b & c: XYF $n=10$. Panels d–f: 2XF $n=12$, 2XM $n=16$. Panel c: 1XF $n=7$, 1XM $n=17$. Panels e & f: 1XF $n=6$, and 1XM $n=13$.

Fig. 3.

Y* mice with two copies of the X-chromosome mount and thrust at a faster rate during individual trials than mice with one copy of X. Mean $\pm$ SEM for mounting and thrusting rates in adult testosterone treated ovariectomized females (OVX+T) and castrated males (Cast+T) tested with receptive females; mounting and thrusting rates were calculated after the first mount and thrust, respectively, only in individual trials in which the behaviors occurred. (a–b) Histograms of Four Core Genotypes (FCG) behaviors. XX, XX genotype (black bars). XY, XY genotype (white bars). (c–d) Histograms of Y* behaviors. 2X, two X-chromosomes genotype (black bars). 1X, one X-chromosome genotype (white bars). (a) FCG mice did not differ in mounts per minute. (b) FCG males had a faster rate of thrusts per minute than females. (c) 2X Y* mice had a faster rate of mounts per minute. (d) 2X Y* mice, and males, had faster rates of thrusts per minute than 1X mice, and females, respectively. **Significant difference between mice with two X-chromosomes versus one ($P < 0.01$). #Significant gonadal sex difference ($P < 0.05$). Panels a & b: XX females (XXF) $n = 11$, XX males (XXM) $n = 11$, and XYM $n = 9$. Panel a: XYF $n = 11$. Panel b: XYF $n = 10$. Panels c & d: 2XF $n = 12$, and 2XM $n = 16$. Panel c: 1XF $n = 7$, and 1XM $n = 17$. Panel d: 1XF $n = 6$, and 1XM $n = 12$.

Fig. 4.

Male Y* mice with two X-chromosomes show trends for increased aggression toward an intruder than males with a single X-chromosome. **(a)** Kaplan-Meier curves for the percent accumulation of latency to attack an intruder-male over three, ten-minute trials. **(b)** Histograms of the number of mice attacking in 0 (white bars), 1 (lined bars), or 3 (black bars) trials. **(a)** Males with two copies of X (2XM, solid line), showed a trend for attacking an intruder faster than males with one X (1XM, dashed line; $P=0.07$). **(b)** 2XM showed a trend for attacking in more trials than 1XM ($P=0.1$). Cast+T, adult testosterone treated castrates. 2XM, $n=20$. 1XM, $n=20$.

Fig. 5.

Y* male mice have a larger density of vasopressin immunoreactive area in the lateral septum than females. Mean $\pm$ SEM μm^2 immunoreactive area. 2X, two X-chromosomes genotype (black bars). 1X, one X-chromosome genotype (white bars). OVX+T, testosterone treated ovariectomized females. Cast+T, adult testosterone treated castrated males.

#Significant gonadal sex difference ($P < 0.05$). In all groups $n=9$.

Fig. 6.

Y* mice show no differences in gene expression of the androgen receptor (*Ar*), aromatase (*Cyp19a1*), or estrogen receptor- α (*Esr1*) in the preoptic area of the hypothalamus (POA). Mean $\pm$ SEM relative quantification (RQ) of: (a) *Ar*, (b) *Cyp19a1*, and (c) *Esr1*. RQ values were calculated using the $\Delta\Delta C_t$ method with cyclophilin B (*Ppib*) as the endogenous control and normalized to the mean of 1X males. 2X, two X-chromosomes genotype (black bars). 1X, one X-chromosome genotype (white bars). OVX+T, adult testosterone treated ovariectomized females. Cast+T, adult testosterone treated castrated males. In all groups $n=6$.

Table 1
Genotype and sex chromosome complement of FCG and Y* mice

The gonadal sex and dose of sex chromosomes are shown among genotypes of the Four Core Genotypes (FCG) and Y* models. In the genotypes column, the first X represents the maternally inherited sex chromosome, and the second sex chromosome is paternally inherited. Y⁻, Sry deleted Y-chromosome. Sry, sex-determining region of Y (FCG Sry is a transgene within an autosome). Copies of X, dose of X-chromosome specific genes. Copies of Y, dose of Y-chromosome specific genes.

Composition of Sex Chromosome Regions

	Genotype	Gonads	Sry	Copies of Y	Copies of X
FCG					
XYM	XY ⁻ Sry	Testes	1	1	1
XXM	XXSry	Testes	1	0	2
XYF	XY ⁻	Ovaries	0	1	1
XXF	XX	Ovaries	0	0	2
Y*					
1XM	XY*	Testes	1	1	1
2XM	XX ^{Y*}	Testes	1	1	2
1XF	XY ^{*X}	Ovaries	0	0	1
2XF	XX	Ovaries	0	0	2

Table 2
Oligonucleotide primers used for qRT-PCR

Nucleotide sequence of primers used to amplify the mRNA transcripts of the genes *Ar* (androgen receptor), *Cyp19a1* (aromatase), *Esr1* (estrogen receptor- α), and cyclophilin B (*Ppib*). Accession numbers are from the NCBI Reference Sequence database. Forward (Fwd.) and reverse (Rev.) primers anneal to cDNA antisense and sense strands, respectively. Numbers in parentheses indicate the primers' 5'-nucleotide position on the reference sequence.

Forward and reverse oligonucleotides primers of *Ar*, *Cyp19a1*, *Esr1*, and *Ppib*.

Transcript	Accession #	Nucleotide sequence (5' position)
<i>Ar</i>	NM_013476.3	Fwd. 5'-AGAATCCCACATCCTGCTCAA (2512) Rev. 5'-AAGTCCACGCTACCATATGG (2644)
<i>Cyp19a1</i>	NM_007810.3	Fwd. 5'-ACCTCGGGCTACGTGGATGTG (582) Rev. 5'-GATGTTGGTTTGATGAGGAGAGC (740)
<i>Esr1</i>	NM_007956.4	Fwd. 5'-AATTCTGACAATCGACGCCAG (650) Rev. 5'-GTGCTTCAACATTCTCCCTCCTC (994)
<i>Ppib</i>	NM_011149.2	Fwd. 5'-TGGAGAGCACCAAGACAGACA (642) Rev. 5'-TGCCGGAGTCGACAATGAT (707)

Table 3
Testosterone levels in adult Y* mice

Mean $\pm$ SEM ng/ml testosterone in serum (numbers per group). Gonad intact Y* males had higher average testosterone concentrations than gonad intact Y* females, but testosterone levels did not significantly differ between males with one or two doses of the X-chromosome, or females with one or two X-chromosomes. Testosterone concentrations did not differ between any genotype after gonadectomy and T-implant treatment. 1XM, males with one copy of X. 2XM, males with two copies of X. 1XF, females with one copy of X. 2XF, females with two copies of X.

Mean $\pm$ SEM adult serum testosterone levels (number of mice).

Genotype	Gonad-Intact T (ng/ml)	GDX + T-implant (ng/ml)
1XM	3.64 $\pm$ 1.64 (19)	4.84 $\pm$ 0.29 (20)
2XM	2.12 $\pm$ 0.83 (21)	4.35 $\pm$ 0.26 (19)
1XF	1.52 $\pm$ 0.54 (18)	5.83 $\pm$ 0.47 (11)
2XF	0.82 $\pm$ 0.47 (19)	5.13 $\pm$ 0.33 (11)