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Feminized Behavior and Brain Gene Expression in a Novel Mouse Model of Klinefelter Syndrome

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Abstract

Klinefelter Syndrome is the most common sex chromosome aneuploidy in men and is characterized by the presence of an additional X chromosome (XXY). In some Klinefelter males, certain traits may be feminized or shifted from the male-typical pattern towards a more female-typical one. Among them might be partner choice, one of the most sexually dimorphic traits in the animal kingdom. We investigated the extent of feminization in XXY male mice (XXYM) in partner preference and gene expression in the bed nucleus of the stria terminalis/preoptic area and the striatum in mice from the Sex Chromosome Trisomy model. We tested for partner preference using a three-chambered apparatus in which the test mouse was free to choose between stimulus animals of either sex. We found that partner preference in XXYM was feminized. These differences were likely due to interactions of the additional X chromosome with the Y. We also discovered genes that differed in expression in XXYM vs. XYM. Some of these genes are feminized in their expression pattern. Lastly, we also identified genes that differed only between XXYM vs. XYM and not XXM vs. XYM. Genes that are both feminized and unique to XXYM vs. XYM represent strong candidates for dissecting the molecular pathways responsible for phenotypes present in KS/XXYM but not XXM. In sum, our results demonstrated that investigating behavioral and molecular feminization in XXY males can provide crucial

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information about the pathophysiology of Klinefelter Syndrome and may aid our understanding of sex differences in brain and behavior.

Keywords

sexual orientation; Klinefelter Syndrome; sexual behavior; 47, XXY; sex chromosome trisomy; partner choice

Introduction

Klinefelter Syndrome (KS) is characterized by the presence of an extra X chromosome in men resulting in a karyotype of 47, XXY. It has a frequency of 1:426 to 1:1000, making it the second most frequent chromosomal aneuploidy in live births after trisomy 21 (Down's Syndrome) and the most frequent sex chromosome aneuploidy in humans (Bojesen, Juul, & Gravholt, 2003; Egan et al., 2004; Maclean, Harnden, & Court Brown, 1961; Robinson, Bender, Linden, & Salbenblatt, 1990; Smyth & Bremner, 1998). Men with KS (henceforth "KS men") experience hypogonadism and are almost always infertile. Testosterone (T) levels are significantly lower than non-KS men, starting at puberty (Smyth & Bremner, 1998).

Several traits in KS men are feminized (i.e., they are shifted from the male-typical pattern towards a more female-typical one). Firstly, KS men tend to have a body fat distribution more akin to women and many experience gynecomastia (Smyth & Bremner, 1998). Secondly, numerous lines of evidence indicate that being XXY may be a risk factor for other autoimmune diseases, which are typically strongly female-biased (Oktenli et al., 2002; Rovensky, Imrich, Lazurova, & Payer, 2010). For instance, the risk of systemic lupus erythematosus (SLE) in KS men is ~14-fold higher than in 46, XY men—a relative risk similar to that of 46, XX women (Scofield et al., 2008). Thirdly, there is an increased rate of homosexual behavior and gender non-conformity among KS males (Bancroft, Axworthy, & Ratcliffe, 1982; Schiavi, Theilgaard, Owen, & White, 1988; Theilgaard, 1984). For instance, KS boys have more problems relating to other boys, are less likely to show stereotypically masculine interests, and are less sexually interested in girls compared to their 46, XY counterparts—which could be a consequence of lower T levels at puberty (Bancroft et al., 1982; Ratcliffe, Bancroft, Axworthy, & McLaren, 1982). Similarly, KS men feel more negatively about conventional gender roles during childhood, judged as less masculine by others, and have poorer self-esteem in adulthood compared to control males (Schiavi et al., 1988). Finally, more KS men engage in sexual acts with other men compared to non-KS men (Schiavi et al., 1988; Theilgaard, 1984). Altogether, partner choice also appears to be feminized in KS men.

A greater understanding of the factors that lead to the phenotypes associated with KS is crucial for better clinical management. This information could also benefit our knowledge concerning sex differences in neurological disease, cognition, and behavior. Differences between KS and XY men can be ultimately traced to the presence of the extra X chromosome, the potentially lower levels of androgens starting at puberty, or the interaction of these two factors. At present, however, the pathophysiology of KS is still poorly

understood. The dissociation of hormonal vs. chromosomal causes of the KS phenotype would be aided by an animal model.

In this article, we describe the results of experiments performed on a novel mouse model of KS called the Sex Chromosome Trisomy (SCT) model (Chen et al., 2013). In SCT mice, gonadal sex is decoupled from sex chromosome complement. In brief, we are able to generate animals with the following genotypes: XX, XY, XXY, and XYY, all of which can be gonadally male (M, with testes) or female (F, with ovaries) (see Fig. 1A and Method section for more details). We have focused on XYM, XXM, XXYM, and XXF for this study.

Other mouse models of KS have been reported (Swerdloff, Lue, Liu, Erkkila, & Wang, 2011; Wistuba et al., 2010). The first involves a four-generation breeding scheme that produces C57BL/6 XXY and XY mice in the same litters (Swerdloff et al., 2011). Although this model has been used fruitfully to discover important phenotypes associated with the XXY genotype in mice, the breeding scheme is challenging. The second model uses commercially available C57BL/6 XY* mice and has an easier breeding scheme (Wistuba et al., 2010). A fuller explanation of the advantages of the SCT model can be found in a recent article from our group (Chen et al., 2013).

We tested the hypothesis that XXYM from the SCT model are more feminized than XYM in terms of partner preference. Sexual orientation or partner choice is one of the most sexually dimorphic behaviors in the animal kingdom (Ngun, Ghahramani, Sánchez, Bocklandt, & Vilain, 2011). However, little is known about the molecular basis of partner choice. The existing evidence strongly implicates the X chromosome as playing a role in male sexual orientation. A study on a different mouse model of KS found that XXY males, like their XY counterparts, prefer odors from estrous females compared to those from males (Liu et al., 2010).

In this study, we used live animals as stimuli to supply a richer and fuller set of cues relevant to this behavior. We also measured gene expression in the combined bed nucleus of the stria terminalis and preoptic area (BNST/POA) and the striatum, with the hypothesis that expression in XXYM, relative to XYM, would be feminized or shifted towards that of XXF mice. Both of these regions are known to be involved in several sexually dimorphic traits and to be responsive to the actions of both gonadal hormones and sex chromosome complement.

Method

Animals

All experimental procedures using mice were approved by the UCLA Chancellor's Animal Research Committee. All mice used in this study were from the MF1 outbred strain and were bred in the Life Science Vivarium at UCLA. The initial MF1 stocks were a gift from Dr. Paul Burgoyne, MRC National Institute for Medical Research, London. The mice were kept at a 12:12 light:dark cycle with food and water available *ad libitum*. In the SCT model, gonadal sex (whether the animal has testes or ovaries) is uncoupled from the presence/

absence of the Y chromosome. A spontaneous mutation on the Y chromosome led to the creation of the Y^- chromosome that is missing the testis-determining gene, *Sry* (Lovell-Badge & Robertson, 1990). In some mice, *Sry* is present as a transgene inserted into an autosome. Given the many generations of breeding of this line, it is unlikely that there is more than one transgenic insertion site. The number of copies of *Sry* at the insertion site is unknown. Animals that lack this transgene develop ovaries and are gonadally females. Those with the *Sry* transgene develop testes and are gonadally males.

In the SCT model, XXY^- females are crossed to $XY^-(Sry+)$ males. XXY^- females produce X and XY^- eggs whereas $XY^-(Sry+)$ males produce four types of sperm: X, $X(Sry+)$, Y^- , and $Y^-(Sry+)$. Therefore, offspring had one of four sex chromosome complements (XX , XX^- , XXY^- , or XY^-Y^-) each with or without the *Sry* transgene. In this study, we focused on only $XX(Sry+)$ gonadal males, XX gonadal females, $XY^-(Sry+)$ gonadal males, and $XXY^-(Sry+)$ gonadal males. Mice in each of these groups came from 8-11 different litters. The first XXY^- mother stemmed from a cross of an XX female and with $XY^-Y^{*X}(Sry+)$ male (for details of the Y^* model, please refer to Burgoyne, Mahadevaiah, Perry, Palmer, & Ashworth, 1998; Chen et al., 2008; Eicher et al., 1991, but they can also be made from XY^- mothers. The genotype of offspring was determined from karyotype of metaphase spreads of skin fibroblasts (Itoh & Arnold, 2005).

Procedure

Surgery, Hormone Replacement, and Tissue Dissection—All mice used in this study were gonadectomized bilaterally between 97 and 124 days of age. At the time of gonadectomy, a Silastic capsule (1.57 mm inner diameter $\times$ 2.41 mm outer diameter filled to 5 mm in length, sealed at both ends with Silastic polymer) with crystalline testosterone was implanted subcutaneously into the neck of each mouse. Following surgery, each animal was housed individually. We gonadectomized after puberty, so that all genotypes would have the chance to experience the effects of puberty fully. We avoided gonadectomy near to the time of puberty, in case the timing of puberty differed according to genotype. In addition, *a priori*, it is not clear whether the effects of sex chromosome complement are exerted or most obvious when gonadal hormones are high or low. Gonadectomy and the subsequent testosterone administration attempts to remove the normal XXY vs. XY difference in the level of circulating androgens in adulthood, to measure genotypic effects that are not confounded by differences in the acute effects of androgens at this time of life.

Tissue collection was performed 4 weeks after gonadectomy (between 125 and 152 days of age) (Fig. 1C). Mice were anesthetized with isoflurane and then immediately decapitated. Whole brain was rapidly removed from the skull and brain regions of interest were dissected under a microscope, ventral side down on an ice-cold slide. After removal of the dura mater, two cuts through the brain along the coronal plane were made: (1) the midpoint of the optic chiasm (0.14 mm anterior to bregma) and (2) where the optic tract enters the brain (0.58 mm posterior to bregma). The resulting slab of tissue was placed posterior side down. The BNST/POA was defined as the region ventral to the lateral ventricle and bounded laterally by the medial edge of the internal capsule. The striatum was defined as the tissue between the external capsule and the anterior commissure, bounded laterally by the cortex and

medially by the internal capsule. After dissection, the tissue was immediately placed on dry ice and stored at -80C until it was processed.

Testosterone Assay—Blood was obtained from the carotid artery following decapitation. Serum was then isolated and stored at -20C until assays for testosterone were performed. Testosterone assays using radioimmunoassay were performed by Ligand Assay and Analysis Core at the University of Virginia Center for Research in Reproduction (supported by NICHD [SCCPIR] Grant U54-HD28934). Testosterone measurements were performed in singlet reactions using Siemens Medical Solutions Diagnostics testosterone RIA with a reportable range of 0.72-111.00 ng/L. There were no significant differences in measured testosterone levels between our experimental groups when analyzed by ANOVA: $F(5, 70) = 1.53$.

Partner Preference Testing—We used 8-13 animals from each genotype of interest in our partner preference tests. All animals were sexually naïve. For a schematic of the testing apparatus, see Fig. 1B. All preference tests were conducted in a Plexiglas box measuring $8 \times 8 \times 36$ in. At each lateral end of the apparatus, a clear Plexiglas divider with evenly-spaced $\frac{1}{2}$ -in. air-holes was inserted to create a chamber measuring 4×8 in. Each end chamber housed a single stimulus animal. Contact between the stimulus and test animals was thus prevented but auditory, visual, and olfactory cues could be communicated. The area 0-5 in. away from the stimulus animal's chamber was defined as the “incentive zone” (Paredes, 2009).

Stimulus males were left gonadally intact and were sexually experienced. All stimulus females were ovariectomized and injected subcutaneously 48 and 24 hr before testing with estradiol benzoate (1.25 mg/kg mouse; dissolved in sesame oil) followed by progesterone (1 mg per mouse; dissolved in sesame oil) 3 hr before testing to induce estrous. All behavioral tests were performed right after the end of the dark cycle, between 9:00 a.m. and 1:00 p.m. In order to avoid possible end bias, the sex of the stimulus animal at each end was randomized between test animals.

The testing took place one to two weeks following gonadectomy and testosterone replacement. All animals were singly housed at the time of testing. At the beginning of each test, the test animal was placed in the testing apparatus in the absence of stimulus animals for 10 min to adapt to the testing apparatus and to ensure that there was no development of any end preferences. The stimulus animals were then placed into the side chambers, one sex on each side. Experimenters left the room and the test animal's behavior was recorded for 5 minutes using a video camera.

The test animal was allowed to roam the testing apparatus freely and to choose between the two stimulus animals or spend its time in the large middle compartment away from the stimuli. We quantified the time that the test animal spent within each incentive zone and used this as our measure of time spent with that stimulus animal. The amount of time that all four limbs of the test animal were within the incentive zone was recorded. In addition, the number of times the test animal crossed the incentive zone marker was counted. The

observer was masked to the identity of the test animals. A random selection of tests was re-scored by an independent observer, and the accuracy of scoring was confirmed.

The position of the stimulus animals was varied between tests. After each round of testing, the apparatus was cleaned thoroughly with 70% ethanol and dried thoroughly.

Microarray Processing—We analyzed 7-12 independent RNA samples from each genotype, each sample from a different mouse. Samples were analyzed in two batches. The first batch consisted of 4-6 animals per group and the second batch was made up of 3-6 more animals per group. All samples that were included were from mice that had undergone the partner preference testing described earlier. Gene expression was surveyed using the MouseRef-8 v2.0 Expression BeadChip Kit (Illumina, catalog no. BD-202-0202). Total RNA was isolated from the BNST/POA and striatum using Qiagen AllPrep DNA/RNA Mini Kit (catalog no. 80204). cDNA synthesis, labeling, and hybridization steps were performed by the UCLA Neuroscience Genomics Core (<http://www.semel.ucla.edu/ungc/services>).

Quantitative Reverse Transcription–Polymerase Chain Reaction (qRT-PCR)—Reverse transcription was performed using the Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA, catalog no. BIO-65043) with 500 ng of total RNA as template. The RNA samples used for validation were from the original microarray samples contingent on availability ($n = 6-8$ per genotype). The primer sequences used are detailed in Table 1. All primers used spanned at least one intron. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of gene expression between samples. qRT-PCRs were carried out in duplicate utilizing the Syber Green-based SensiMix SYBR No-Rox Kit (Bioline, catalog no. QT650-05). For all reactions, the cycling conditions were as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 sec, 62°C for 15 sec, and 72°C for 15 sec.

Statistical Analyses

Partner Preference and Microarray Data Processing—For partner preference testing, XXYM were first compared to XYM. Relative preference for one stimulus sex within a group (whether that genotype spent more time with the stimulus male or the stimulus female) and comparisons of time spent with each stimulus animal between XXYM and XYM were determined using the two-tailed Mann-Whitney U test. Effect size of the difference between XXYM and XYM in time spent with each stimulus sex, r , was estimated by $Z/\sqrt{N}$ (Field, 2005). When XXM were included in comparisons alongside XYM and XXYM, we used the two-tailed Kruskal–Wallis one-way ANOVA. Microarray data were processed using the statistical software program R (<http://www.r-project.org/>). Data were first background corrected using the *nec* function in the *limma* package (Haga et al., 2010). Probes with low-quality data (detection p -value of $> .05$ in more than a third of the samples) were then removed from the dataset. Next, batch effects were corrected using the ComBat R script and the data underwent quantile normalization and $\log_2$ transformation using the *limma* package (Johnson, Li, & Rabinovic, 2007). The data have been submitted to GEO (accession numbers: GSE48783 and GSE48784). qRT-PCR was used to validate the microarray results. We used the standard curve method to determine relative expression and

assessed significance using the Student's *t*-test ($\alpha = .05$). Data were expressed as fold change where the expression level in XYM has been set to 1.

Determination of Feminized Genes in XXYM—We first established a class of genes that differed in expression between XXF and XYM, using non-stringent criteria ($p \leq .05$ in a two-tailed Student's *t* test, and fold change ≥ 1.2) to assess genes that were differentially expressed in XXF vs. XYM. These genes were called “sexually dimorphic” in their expression (Shi et al., 2008; Wijchers et al., 2010).

To determine whether the expression level in XXYM was feminized (i.e., more similar to XXF than to XYM), we calculated a feminization score. The mean expression value of each gene in the sexually dimorphic class was rescaled so that it equaled 0 in XYM and 100 in XXF. We converted the expression values using the following formula:

$$y = Ax + B$$

where y = the rescaled $\log_2$ expression value (0 = XYM, 100 = XXM), x = normalized $\log_2$ expression value, A = value of slope relating the rescaled value to the original $\log_2$ expression value, B = y -intercept of the slope relating the rescaled value to the original $\log_2$ expression value. Once A and B were determined, the rescaled expression value for that gene in XXYM was calculated. This resulted in a feminization score for each sexually dimorphic gene in XXYM. Scores were capped such that $-25 \leq y \leq 125$.

In order to determine a lower threshold to be considered a candidate feminized gene, we looked at the feminization scores of X chromosome genes that escape inactivation. As XXF and XXYM both have two X chromosomes, we would expect these X inactivation escapees to be both sexually dimorphic and feminized (Yang, Babak, Shendure, & Disteche, 2010). Of the known X inactivation escapees in mice, we found 5 in our set of sexually dimorphic genes in both brain regions (*Kdm6a*, *Mid1*, *Xist*, *2610029G23Rik*, and *Eif2s3x*). We chose a score of 70 as the lower threshold for considering a gene a candidate for feminization in XXYM, as that was the lowest score among the set of X inactivation escapees that we detected. Feminized gene candidates then had their feminization score evaluated using a one-sample *t*-test (H_0 : expected feminization score is 0) and corrected for multiple testing using the Benjamini-Hochberg method (FDR = 10%) (Benjamini, Drai, Elmer, Kafkafi, & Golani, 2001). The heat map of expression patterns of feminized genes that passed the FDR cutoff was generated in R using the heatmap.2 function from the gplots package (Warnes, Bolker, & Lumley, 2013).¹

Determination of Genes Affected by Being XXY—We assessed genes that were expressed differently in XXYM vs. XYM and XXM vs. XYM, using a cutoff ≥ 1.2 fold and $p \leq .05$ (two-tailed Student's *t*-test). These criteria were not meant to adjust for the multiple testing but were used to allow us to rank the most differentially expressed genes between the groups allowing for both effect size and statistical significance. Once the difference

¹Script available on request

categories were established, we used GeneVenn (<http://genevenn.sourceforge.net/>) (Pirooznia, Nagarajan, & Deng, 2007) to determine overlap between the two gene sets. The data were then analyzed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

Results

Partner Preference in XY Males

To test for partner preference in our mice, we used a three-chambered apparatus (Fig. 1B). The results of these tests are summarized in Table 2. To validate these tests in this experimental setup, we tested whether we could detect the expected preference for estrous females in XYM (Bakker, Honda, Harada, & Balthazart, 2002; Liu et al., 2010). As expected, XYM spent significantly more time with the stimulus estrous female than with the stimulus male ($U = 39$, $p = .02$ by Mann-Whitney; Table 2).

Partner Preference in XXY and XX Males

We first compared XXYM to XYM as this is a clinically relevant dyadic comparison. XXYM did not show a preference for the estrous female. Instead, there was a weak trend for XXYM to spend more time with the stimulus male than with the stimulus female ($U = 46$, $p = .28$ by the Mann-Whitney test; Table 2).

We then analyzed time spent with each stimulus sex separately. Based on the higher rates of homosexual behavior seen in KS men, we hypothesized that XXYM would spend less time with the stimulus female and a greater amount of time with the stimulus male when compared to XYM. We found that part of this hypothesis was borne out. XXYM spent significantly less time with the stimulus female than XYM ($U = 23$, $p = .036$ by Mann-Whitney; effect size, $r = -.46$; Fig. 2A). The difference in time spent with the stimulus male was not significantly different between XXYM and XYM ($U = 35$, $p = .24$, by Mann-Whitney; $r = .27$; Fig. 2B). Based on the time spent with the female, these results suggest that XXYM are less attracted to estrous females.

We then compared XYM, XXM, and XXYM to each other. XXM did not show a clear preference for estrous females and spent similar amounts of time with both stimulus animals of both sexes ($U = 36$; Table 2). XXM were not significantly different from the other groups in either time spent with the stimulus male or female based on the Kruskal-Wallis test.

Effect of Genotype on Motor Behavior

Klinefelter patients show deficits in their motor skills, including running speed, visual motor control, response speed, and motor speed (Boone et al., 2001; Ross et al., 2008; Ross, Zeger, Kushner, Zinn, & Roeltgen, 2009). Therefore, a potential source of the observed differences in partner preference between XYM and XXYM may be due to deficits in motor function. To test the possibility of differences in locomotor function, we measured the number of approaches the test animal made to the stimulus animals. An approach was defined as each time the test animal entered the incentive zone. No significant between-group differences were seen in the number of approaches made to the stimulus female, stimulus male or the

overall total (Table 3). Additionally, there were no significant within-group differences in number or approaches to the female vs. the male. The lack of difference between XXY vs. XY mice in motor performance was confirmed using the challenging beam traversal test and the pole test, which are sensitive and standard tests of motor function. There were no significant differences between XYM and XXYM on these tests (Chen et al., 2013).

Assessment of the Degree of Feminization of Gene Expression in the BNST/POA and Striatum of XXY Males

We then asked if the gene expression profile in XXYM male mice was feminized and, if so, what the extent of this feminization was. We examined two regions of the brain that display sexual dimorphisms: the striatum and the BNST/POA. Genes classified as sexually dimorphic (different between XYM and XXF) comprised 216 genes in the BNST and 364 genes in the striatum (Supp. Table 1), with 18 genes found to be sexually dimorphic in both regions. The direction of difference was the same in both tissues in 16 of these genes. The two exceptions were *Chga* and *Amigo1*, both of which were higher in XXF than XYF in the BNST/POA but in the opposite direction in the striatum.

We rescaled the expression values for each of the 562 sexually dimorphic genes as described in the Method. If that gene received a score of 70 or higher, it was deemed a candidate for feminization. We chose 70 as a cutoff because this was the lowest score of a sexually dimorphic, known X-inactivation escapee (*Utx*) in our dataset.

The expression patterns of the majority of sexually dimorphic genes in XXYM more closely resembled XYM than XXF (153 of 216 genes score below 50 in the BNST/POA, 270/364 in the striatum) so gene expression in XXYM in both brain regions was not generally feminized (Fig. 3A and C). However, a substantial minority of genes scored 70 or above (30 in the BNST/POA; 36 in the striatum).

We then performed a one-sample *t*-test on the feminization scores followed by Benjamini-Hochberg correction (FDR = 10%) on this list. Genes that survived this correction were deemed feminized. This resulted in 27 of 216 sexually dimorphic genes in the BNST/POA being categorized as feminized genes whereas 24 out of 364 sexually dimorphic genes in the striatum qualified. The expression patterns of these feminized genes in XXYM, XYM, and XXF are visualized as heat maps in Fig. 3B and D. 5 genes were common to the list of feminized genes in the two regions: *2610029G23Rik*, *Eif2s3x*, *Kdm6a* (a.k.a. *Utx*), *4933439C20Rik*, and *Xist*. The first four are known X-inactivation escapees and *Xist* is critical for the silencing of the inactive X chromosome (Yang et al., 2010). Taken together, these data imply that the process of X-inactivation occurs normally in XXYM in the BNST/POA and striatum.

Differential expression of two feminized genes was verified using qRT-PCR. Those results were consistent with the microarray data (Fig. 4).

Differences in Gene Expression between XXY and XY in the BNST/POA and Striatum

We were also interested in determining the effect of the additional X chromosome in XXYM on gene expression in the BNST/POA and striatum and to investigate if there were

differentially expressed genes between XXYM and XYM beyond the feminized ones. Analysis of gene expression between these two genotypes revealed a large number of genes that were differentially expressed ($p < .05$; fold > 1.2). In the BNST/POA, there were 190 differentially expressed genes. 29 genes were more highly expressed in XXYM than in XYM, whereas 161 genes had lower expression (Supp. Table 2, BNST_POA tab). In the striatum, there were 69 differentially expressed genes—17 were higher in XXYM and 52 were lower (Supp. Table 2, Striatum tab). Many of the genes that were detected as differentially expressed were autosomal. Only seven genes were found to be differentially expressed in both the BNST/POA and the striatum. All of these genes were more highly expressed in XXYM than XYM in both tissues.

We used Ingenuity Pathway Analysis to functionally annotate these differentially expressed genes and to characterize the pathways that were different between XXYM and XYM. In the BNST/POA, 8 of the top 10 pathways affected by being XXYM vs XYM were related to immune function (Table 4). In the striatum, although pathways were more varied, immune function-related pathways were still dominant as they made up 5 of the top 10 pathways (Table 5).

Because intact XXYM mice have lower basal testosterone levels than XYM (Lewejohann et al., 2009; Lue et al., 2005), the differences in gene expression between XXYM and XYM could be either downstream of group differences in levels of testicular hormones prior to gonadectomy or could be more related to sex chromosome complement, including the interaction of the second X chromosome and a Y chromosome. To elucidate this issue, we compared the XXYM vs. XYM difference in gene expression to differences in expression in XXM vs. XYM, which are not known to have differences in hormone levels, and which also involve male groups with one vs. two X chromosomes. In the BNST/POA, 164 genes were differentially expressed between XXM and XYM (Supp. Table 3, BNST_POA tab). In the striatum, 208 genes were different between XXM and XYM (Supp. Table 3, Striatum tab). We compared these differences to those found in XXYM vs. XYM (Fig. 5). We found that of the 190 genes different between XXYM and XYM in the BNST/POA, 170 were unique to that comparison (Fig. 5A, white). In the striatum, 51 of the 70 genes were unique (Fig. 5B, white). Genes that are unique to XXYM vs. XYM (i.e., not found in XXM vs. XYM) are candidates for genes affected by interactions between the additional X and the Y and/or testosterone deficiency. Furthermore, since we only observed a significant difference in partner preference between XXYM and XYM, these uniquely affected genes may be candidates for genes associated with this partner preference phenotype and other traits unique to KS/XXYM.

On the other hand, genes detected as differentially expressed in both comparisons (XXYM vs. XYM and XXM vs. XYM) are likely affected directly by the presence of the second X chromosome—independent of the presence of a Y chromosome. They are also less likely to play a large role in traits found in KS/XXYM but not XXM. 20/170 genes in the BNST/POA are affected by being a 2X male (Table 6 and Fig. 5A, gray). A total of 19 of these genes were affected by being a 2X male (Table 7 and Fig. 5B, gray). In both regions of the brain, the direction of the change of genes affected by being a 2X male almost always matched between the two pairwise comparisons—if a gene was upregulated in XXYM

relative to XYM, it was also upregulated in XXM compared to XYM. The single exception to this was *Dnalc1* in the striatum, which is downregulated in XXYM vs. XYM but upregulated in XXM vs. XYM. *Dnalc1* codes for the light chain of axonemal dyneins and its expression is downregulated in a mouse model of multiple sclerosis (Kreutzer et al., 2012). Some of these 2X male genes are known X-inactivation escapees (for example *Mid1*, *Eif2s3x*, and *Kdm6a*) but most are autosomal.

Discussion

In this study, we present a novel mouse model for the study of sex chromosome aneuploidies termed the SCT model. The SCT model produces XXY, XYY, XY, and XX mice, all with either testes or ovaries. Here, we examined a subset of these groups for specific comparisons relevant to KS. We were particularly interested in partner preference, and found lower preference for female mice in XXYM relative to XYM. We also discovered a significant shift in patterns of gene expression in the brain of XXM, away from those normally found in XYM mice and towards those found in XXF mice.

Some important caveats regarding extension of our data from this mouse model to the human condition should be noted. The mechanisms leading to sexual differentiation of the brain have several key differences between rodents and humans. In rodents, masculinization and defeminization of the brain are mainly due to the actions of pre- and perinatal estradiol, which is produced via aromatization of T (Lenz & McCarthy, 2010). However, in primates, it is the direct action of fetal T that is thought to be critical for brain sexual differentiation (Cohen-Bendahan, van de Beek, & Berenbaum, 2005; Hines, 2011). Furthermore, the neural pathways underlying partner choice also show some species differences. For instance, pheromones play a large role in sexual behavior in mice (Haga et al., 2010; Osada, Hanawa, Tsunoda, & Izumi, 2012) but the existence of human pheromones is still a subject of some debate (Grammer, Fink, & Neave, 2005; Hays, 2003). Finally, the SCT model is unlikely to fully recapitulate all the features of KS.

To test for feminization of partner preference, we used a tri-compartment apparatus that prevents physical contact between the test and stimulus animals. This allowed us to examine just the approach aspect of partner preference behavior (Paredes, 2009). We used live animals as opposed to used bedding, which is the more common choice, to provide a more complex and salient set of partner-cues. The use of live animals, as in our study, enables the communication of visual and auditory cues in addition to olfactory cues found in used bedding, all of which are important components of mating behavior (Bakker et al., 2002; Pomerantz, Nunez, & Bean, 1983). The use of live animals may present some potential confounds because it may introduce social approach and interest components to a test for sexual partner preference. However, approach behaviors are an important part of partner preference and, under naturally occurring conditions, social and sexual components that affect partner preference are always in play simultaneously (Paredes, 2009).

We observed that XXYM spent less time near the stimulus female than XYM. XXM were not significantly different from either XYM or XXYM on any of our measures. This implies that the feminization of partner preference in XXYM is not due solely to the presence of the

additional X chromosome but rather requires interactions between this chromosome and the Y. Furthermore, we did not detect any differences in partner preference between XXYY and XYF (data not shown). This result suggests that the differences observed between XXYY and XYM requires the presence of testes at some life stage before the age of gonadectomy. For example, the preference may require perinatal androgenization. This finding is in contrast to a recent study that found that mice with two X chromosomes, compared to those with one X chromosome, showed an increase in male copulatory behaviors (mounting, thrusting, and ejaculation) independent of gonadal sex, perinatal androgenization, and the presence of the Y chromosome (Bonthuis, Cox, & Rissman, 2012). Therefore, it appears that the effect of an additional X chromosome may be different between partner preference (where it appears to feminize in conjunction with the Y) and copulation (where it masculinizes the behavior independent of the Y). This difference is not unexpected as approach (partner preference) and consummation (copulatory behavior) are distinct aspects of sexual behavior and may be regulated differently.

There may have been differences in hormonal levels between XXYY and XYM prior to gonadectomy that could have caused differences in the organization of brain regions relevant to this behavior and led to its subsequent feminization in XXYY relative to XYM. Lower levels of testosterone have been detected in XXYY, as compared to XYM, in two other mouse models of KS (Lewejohann et al., 2009; Lue et al., 2005). The role of androgen and its metabolites in establishing partner preference in mice is demonstrated by the abolishment of a preference for odor from estrous females in male mice lacking functional aromatase (Bakker et al., 2002). It is important to note that the behavioral differences that we observed are very unlikely to have arisen from group differences in the level of androgens at the time of testing, because all test animals underwent gonadectomy and received equivalent testosterone implants. There were no significant differences in the levels of testosterone following surgery.

A recent study examined sex preference in a different mouse model of KS (Liu et al., 2010). Liu et al. found that XXYY and XY male mice that were castrated as adults and received testosterone (a treatment paradigm similar to ours) preferred odors from estrous females over those from males. The difference in the two studies could stem from our use of live stimulus mice, in contrast to bedding used by Liu et al. Another possible source of the difference between the studies is the genetic background of the mice used (the inbred C57BL/6J in the Liu et al. study and the outbred MF1 in ours), which can cause differences in sexual behavior (Dominguez-Salazar, Bateman, & Rissman, 2004). Liu et al. also concluded that it was social rather than sexual traits that were affected in XXYY males because they prefer male odors over those from ovariectomized females. If that is also the case with SCT mice, it would appear that a social preference for male mice is able to overcome the sexual cues from the receptive stimulus female when a live animal is used as a stimulus. Finally, there was a difference in the choice of testing time. We performed our behavioral testing shortly after the end of the dark phase of the light:dark cycle but Liu et al. performed their tests in the afternoon, several hours after their dark phase ended. Future studies on social traits in SCT mice will greatly contribute to the interpretation of our data.

In the second part of this study, we investigated the extent of feminization of gene expression in the BNST/POA and striatum of XXYM relative to XYM. The BNST and POA are both subject to long-lasting, irreversible changes due to perinatal testosterone exposure (Ngun et al., 2011). The POA is implicated in the regulation of male copulatory behavior and has been linked to male-typical patterns of partner preference (Ngun et al., 2011). The BNST is involved in the control of male sexual behavior, gonadotropin release, and the modulation of stress, all of which show large sex differences (Ngun et al., 2011). The striatum, a region involved in dopaminergic function and reward, displays several key sex differences, many of which are caused by gonadal steroid hormones such as estrogen. For instance, many aspects of dopamine metabolism are influenced by estrogen (Becker, 1999; Davis, Davis, & Halaris, 1977; Fernandez-Ruiz, Hernandez, de Miguel, & Ramos, 1991; Morissette & Di Paolo, 1993). Additionally, a study from our lab showed that *Sry* (the Y-linked male sex determination gene) enhanced striatum DA release and regulated sensorimotor functions of dopaminergic neurons (Dewing et al., 2006).

We found that although gene expression was not generally feminized in XXYM, a substantial proportion of genes were feminized in XXYM in both regions of the brain. Although there was not much overlap between the two regions on the individual gene level, feminized genes in these two regions showed some overlap in molecular functions. Feminized genes in both regions are involved in apoptosis, regulation of cell cycle/proliferation, and neurodevelopment and function. *Snca* or *Park1* was feminized in the striatum only (score of 88.9). It is strongly associated with Parkinson's disease, which not only shows a male bias in incidence but also has gender differences in pathophysiology (Haaxma et al., 2007). Interestingly, women with Parkinson's more often present with tremor, as do KS men (Harlow & Gonzalez-Alegre, 2009). Feminization in expression is likely due to the presence of the additional X chromosome but may also be related to possible differences in androgen levels between XXYM and XYM, prior to gonadectomy.

Interestingly, the set of feminized genes in the BNST/POA was largely made up of those with known neurodevelopmental roles whereas genes that were feminized in the striatum were a mixture of neural development-related genes and those with immune functions. For instance, *Spag9* is feminized in the BNST/POA of XXYM mice (feminization score of 100.2) and seems to have roles in the avoidance of synaptic loss and neurite outgrowth in response to nerve growth factor (Ando et al., 2011; Xu, Dhanasekaran, Lee, & Reddy, 2010). An example of a neurodevelopment gene that is feminized in the striatum of XXYM mice is *Pou3f4* (also called *Brn4*) (feminization score of 71.4), which can induce neural stem cells to differentiate into dopaminergic neurons (Tan et al., 2011). Another feminized striatal gene is *Ifna1* (feminization score of 98.5). It codes for interferon-alpha, which has been linked to the pathogenesis of systemic lupus erythematosus, a strongly female-biased autoimmune disease affecting the brain (Mathian, Weinberg, Gallegos, Banchereau, & Koutouzov, 2005). Emerging evidence indicates that there is crosstalk between the immune, nervous, and endocrine systems (reviewed in Lenz, Nugent, & McCarthy, 2012). Many gender-biased neuropsychiatric traits and disorders such as addiction, autism, schizophrenia, and depression have also been linked to disruptions of the immune system (Crespi & Thiselton, 2011; Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Kelley & Dantzer,

2011). In summary, these data suggest feminization of nervous system and immune genes may play a larger role in the striatum than in the BNST/POA in bringing about differences between XXYM and XYM.

We sought to elucidate biological pathways that were different between XXYM and XYM. In both the BNST/POA and the striatum, immune function pathways were those that were most significantly affected by the second X chromosome in XXY relative to XY. Taken together, our findings hint that differences between XXY and XY males in genes related to the immune system may be responsible for some of the phenotypic divergence between XY and KS men. We hypothesize that there is overlap in the gene pathways used in nervous system development and in immune function and that some of the present nomenclatural distinctions are merely an artifact of which system it was identified in first. An important follow-up to these findings will be to manipulate these pathways *in vivo* in animal models and investigate the consequences on both behavior and brain morphology.

We also identified genes that were differentially expressed between XXYM and XYM but not XXM and XYM. We hypothesize that the genes that are the best candidates for further exploration in the context of traits seen only in KS/XXYM but not XXM will be those that are both feminized and those differentially expressed only between XXYM and XYM. In the BNST/POA, there are four such genes (*Cdc45l*, *Hsd3b2*, *Serpinh1*, and *Thoc3*) whereas there is only one gene in both categories in the striatum (*2310057J16Rik* a.k.a. *Camsap3*). *Hsd3b2* is of particular interest because its gene product is important for the synthesis of several steroid hormones such as progesterone, androstenedione, and testosterone. It is expressed in the brain and polymorphisms in this gene have been associated with paranoid ideation in bipolar disorder (Gottfried-Blackmore, Sierra, Jellinck, McEwen, & Bulloch, 2008; Guennoun, Fiddes, Guezou, Lombes, & Baulieu, 1995; Johansson, Nikamo, Schalling, & Landen, 2012). The expression of this gene is lower in both XXF and XXYM compared to XYM. To our knowledge, there have been no studies on whether synthesis of steroids in the brains of KS men is changed from their XY counterparts. The action of locally synthesized steroids has been likened to neurotransmitters so small changes in local synthesis could potentially have a domino-like effect and lead to dramatic differences in downstream phenotypes (Micevych & Sinchak, 2008). The observation that *Hsd3b2* is feminized only in XXYM and not in XXM indicates that feminization of local synthesis of androgens may be a potential novel mechanism leading to KS phenotypes.

The contribution of sex chromosome complement and number in brain sexual differentiation was the subject of a recent study from Wolstenholme, Rissman, and Bekiranov (2013). They compared gene expression in the brain of developing male embryos from the Y* model with one vs. two copies of the non-pseudoautosomal region of the X chromosome (males: XY* vs. XX^{Y*}) prior to and after gonadal differentiation, which is analogous to our comparison of XYM with XXYM. Wolstenholme et al. found that there were more genes differentially expressed between XY* and XX^{Y*} prior to gonadal differentiation than afterwards, implying that prenatal androgenization can blunt the effect of the additional X chromosome. In line with our results, Wolstenholme et al. found that X chromosome number not only leads to differential expression of known X inactivation escapees but also several autosomal genes. However, aside from the X inactivation escapees, there was little overlap between the

genes that differed between XXY and XY in the present study compared to that of Wolstenholme et al. These differences likely stem from the numerous contrasts between the two studies: (1) regions surveyed (BNST/POA and striatum vs. whole brain) (2) age at which gene expression was investigated (adulthood vs. embryonic), (3) strain of mice (inbred B6 vs. outbred MF1), and (4) genetic model (SCT vs. XY*).

The feminization of several physiological traits in KS men, relative to XY men, has its roots in feminization on a molecular level. Ultimately, this molecular feminization originates from either direct actions of the additional X chromosome or differences in androgen levels between XY and XXY males. None of the existing animal models of KS have been able to disentangle these two factors. The SCT model presents a unique opportunity to examine the separate actions of these factors as well as their interplay. Our findings demonstrated the potential of this model in unraveling the biological forces influencing complex phenotypes. We have shown that interactions between the additional X chromosome and the Y in XXY contribute to the feminization of KS behavioral and molecular phenotypes. Such information is crucial in elucidating not only the pathophysiology of KS, but also the origin of sex differences in brain and behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. The breeding scheme used to generate SCT mice. Mothers are XXY^{-} and produce two types of eggs: X and XY^{-} . Fathers are $XY^{-}(Sry+)$ and make four types of sperm: X , $X(Sry+)$, Y^{-} and $Y^{-}(Sry+)$. The progeny have four possible sex chromosome complements: XX , XXY^{-} , XXY^{-} , or $XY^{-}Y^{-}$ all of which can be with or without *Sry*. **B.** The three-chambered apparatus for partner preference testing. A stimulus animal (either male or an estrous female) was placed in each lateral chamber. The chambers holding the stimulus animals are separated from the large middle chamber by clear, perforated dividers. The test animal is placed in the middle chamber where it is free to choose to spend time close to either stimulus animal (in the gray incentive zones) or by itself in the middle. Time spent in each incentive zone was recorded and used as a measure of time spent with that stimulus animal. **C.** Study timeline. The numbers along the scale are the ages (in days) at which the experimental procedures were carried out. At about postnatal day 100, all mice used in the study underwent bilateral gonadectomy and received an implant of a Silastic capsule filled with testosterone. Behavioral testing began a week after surgery. At about 128 days of age and after completion of behavioral testing, blood and tissue collection was performed.

Figure 2. Time spent with the stimulus animal

The boxplot depicts time spent with the stimulus animal of each sex in quartiles. The whiskers represent the maximum and minimum amount of time spent with the stimulus animal in each group. **A.** XXY, but not XX, males spend significantly less time with the stimulus female compared to XY males. **B.** Median time spent with the stimulus male is higher in XXY compared to XY males. * $p < .05$

Figure 3. Feminization of brain gene expression in XXYM

Levels of expression of genes in XXYM were assigned a feminization score based on the similarity to expression levels in XXF relative to XYM, then plotted on a continuum between average XYM and XXF expression in **A.** the BNST/POA, and **C.** the striatum. Dots represent the mean score of each gene in XXYM whereas lines represent the standard error of the mean. The color of the dot indicates whether expression of that gene is closer to the male (blue) or female (red) end of the spectrum. Genes considered feminized are indicated by the dashed box. A feminization score of 70 was used as the lower boundary for categorizing a gene as feminized. The expression pattern of feminized genes in **B.** the BNST/POA and **D.** the striatum that survive Benjamini-Hochberg correction (FDR=10%) are visualized using a heat map. Genes are clustered based on similarity in their pattern of expression across samples.

Figure 4. Quantitative RT-PCR confirmation of feminized genes in XXY males
mRNA levels of *Eif2s3x* and *Utx* (*Kdm6a*) in XY males, XX females, and XXY males in both the BNST/POA and the striatum were analyzed. Error bars represent the standard error of the mean from 6-8 biological replicates from each group. Expression is relative to GAPDH and is normalized to XY males. * $p < .05$ by the Student's t-test.

Figure 5. Determination of genes affected uniquely by being XXY

The Venn diagram shows genes that are differentially expressed (>1.2 -fold, $p < .05$ by Student's t-test) between XXY and XY males (white), and between XX and XY males (black) in A. the BNST/POA and B. the striatum. This procedure identified 20 genes in the BNST/POA and 19 genes in the striatum that may be different in both comparisons because of the common difference in number of X chromosomes (gray).

Table 1

Primers used in the quantitative RT-PCR validation of microarray results.

Gene	Forward primer	Reverse primer	Product size (bp)
<i>GAPDH</i>	TGCCGCCTGGAGAAACC	CCCTCAGATGCCTGCTTCAC	65
<i>Eif2s3x</i>	TTGTGCCGAGCTGACAGAATGG	CGACAGGGAGCCTATGTTGACCA	198
<i>Kdm6a</i>	CCAATCCCCGCAGAGCTTACT	TTGCTCGGAGCTGTTCCAAGTG	166

Table 2

Median time spent with each stimulus animal in seconds.

Genotype	n	Time spent with stimulus estrous female (s)	Time spent with stimulus male (s)
XYM	13	143 (71.0)	90 (47.0)
XXM	8	121 (65.0)	118.5 (27.8)
XXYM	8	111 (46.5)	140 (63.5)

Note: The interquartile range is given in parentheses. Total length of time of each test is 300 seconds.

Table 3

Median number of approaches to the stimulus animals.

Genotype	n	No. of approaches to stimulus female	No. of approaches to stimulus male	Total no. of approaches
XYM	13	7 (3.5)	7 (3.0)	14 (5.5)
XXM	8	7 (2.5)	8.5 (4.5)	15.5 (5.5)
XXYM	8	7.5 (5.5)	7.5 (4.0)	14.5 (7.5)

Note: Interquartile range is given in parentheses. The number of times the test animal crossed a line into the incentive zone beginning 5 inches away from the stimulus animal's chamber was counted.

Table 4

Top 10 pathways that are significantly different in XXYM vs. XYM in the BNST/POA ($p < 0.05$ Fisher's exact test) as determined by Ingenuity Pathway Analysis.

No.	Ingenuity Canonical Pathway	p-value	Ratio
1	Complement System	3.89E-03	0.086
2	Antigen Presentation Pathway	5.01E-03	0.075
3	Autoimmune Thyroid Disease Signaling	9.33E-03	0.049
4	Graft-versus-Host Disease Signaling	9.33E-03	0.060
5	L-glutamine Biosynthesis II (tRNA-dependent)	9.55E-03	0.091
6	Granzyme B Signaling Cytotoxic T Lymphocyte-mediated Apoptosis of Target	1.02E-02	0.125
7	Cells	1.38E-02	0.039
8	Allograft Rejection Signaling	1.45E-02	0.035
9	IL-4 Signaling	3.24E-02	0.038
10	B Cell Development	3.39E-02	0.061

Note: The list of genes that differ significantly between XXY and XY males was entered into IPA. The 'Ratio' column is the fraction of genes in the input list that are found in that pathway.

Table 5

Top 10 pathways that are significantly different in XXYM vs. XYM in the striatum ($p < 0.05$ Fisher's exact test) as determined by Ingenuity Pathway Analysis.

No.	Ingenuity Canonical Pathway	p-value	Ratio
1	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.16E-03	0.028
2	Phospholipase C Signaling	5.01E-03	0.015
3	Assembly of RNA Polymerase II Complex	9.77E-03	0.036
4	Primary Immunodeficiency Signaling	1.07E-02	0.032
5	CXCR4 Signaling	1.10E-02	0.018
6	Calcium-induced T Lymphocyte Apoptosis	1.51E-02	0.029
7	Clathrin-mediated Endocytosis Signaling	1.86E-02	0.015
8	Thrombin Signaling	2.00E-02	0.015
9	Regulation of Actin-based Motility by Rho	2.34E-02	0.023
10	Salvage Pathways of Pyrimidine Ribonucleotides	2.51E-02	0.020

Note: The list of genes that differ significantly between XXY and XY males was entered into IPA. The 'Ratio' column is the fraction of genes in the input list that are found in that pathway.

Table 6

Genes corresponding to the overlap region (gray) in Fig 5A.

Gene symbol	Accession	Chr	Fold change (XXYM/XYM)	Fold change (XXM/XYM)
<i>Al314976</i>	NM_207219.2	17	0.8	0.8
<i>Ap2b1</i>	NM_001035854.2	11	0.8	0.7
<i>Atp7a</i>	NM_009726.3	X	0.7	0.8
<i>Camkk2</i>	NM_145358.1	5	0.7	0.8
<i>Cendbp1</i>	NM_010761.2	2	0.8	0.8
<i>Cdh11</i>	NM_009866.2	8	0.7	0.7
<i>Echdc1</i>	NM_025855	10	0.7	0.7
<i>Eif2s3x</i>	NM_012010.3	X	1.4	1.4
<i>Erdr1</i>	NM_133362.2	X and Y	2.5	4.9
<i>Gm13212</i>	NM_001013808.1	4	1.3	1.4
<i>Gtf2ird2</i>	NM_053266.1	5	1.3	1.4
<i>Jam3</i>	NM_023277.3	9	0.7	0.8
<i>Kcnq2</i>	NM_001006677.1	2	0.7	0.7
<i>Kdm6a (Utx)</i>	NM_009483.1	X	1.3	1.3
<i>Mid1</i>	NM_183151.1	X	3.7	2.4
<i>Myo9a</i>	NM_173018.2	9	0.8	0.8
<i>Slc5a5</i>	NM_053248.1	8	0.6	0.6
<i>Spag9</i>	NM_001025430.1	11	0.7	0.7
<i>Xist</i>	NR_001463.2	X	2.3	9.2
<i>Zbtb46</i>	NM_027656.2	2	1.2	1.3

Note: All genes had the same direction of change and most had similar fold-change values between the two comparisons (XXYM vs. XYM, XXM vs XYM).

Table 7

Genes corresponding to the overlap region (green) in Fig Venn B.

Gene symbol	Accession	Chr	Fold change (XXYM/XYM)	Fold change (XXM/XYM)
<i>4933439C20Rik</i>	NM_001004146.1	11	1.5	1.4
<i>Apobec1</i>	NM_031159.3	6	0.8	0.8
<i>Atp9b</i>	NM_015805.2	18	0.7	0.6
<i>Bcat1</i>	NM_007532.2	6	0.7	0.8
<i>Dnalc1</i>	NM_028821.1	12	0.8	1.3
<i>Eif2s3x</i>	NM_012010.3	X	1.4	1.6
<i>Ercc2</i>	NM_007949.4	0	1.3	1.3
<i>Erdr1</i>	NM_133362.2	0	1.8	1.8
<i>Mcam</i>	NM_023061.1	9	0.7	0.7
<i>Mid1</i>	NM_183151.1	X	3.3	1.8
<i>Myl6b</i>	NM_172259.1	10	0.8	0.8
<i>Ndor1</i>	NM_178239.2	2	0.8	0.8
<i>Nupr1</i>	NM_019738.1	7	1.2	1.3
<i>Tmem25</i>	NM_027865.1	9	0.7	0.8
<i>Tmem59l</i>	NM_182991.2	8	0.8	0.8
<i>Ttc39a</i>	NM_001145948.1	4	0.7	0.7
<i>Utx (Kdm6a)</i>	NM_009483.1	X	1.3	1.6
<i>Xist</i>	NR_001463.2	X	140.5	127.8
<i>Zap70</i>	NM_009539.2	1	0.7	0.8

Note: Most genes had the same direction of change and similar fold-change values between the two comparisons (XXYM vs. XYM, XXM vs. XYM). The sole exception is *Dnalc1* which is downregulated in XXYM relative to XYM but upregulated in XXM compared to XYM.