



2016

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### Recommended Citation

Link Jenny C., et, al. The importance of having two X chromosomes. *Phil. Trans. R. Soc.* 371 (2016).  
<http://doi.org/10.1098/rstb.2015.0113>

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## Review

**Cite this article:** Arnold AP *et al.* 2016 The importance of having two X chromosomes. *Phil. Trans. R. Soc. B* **371**: 20150113. <http://dx.doi.org/10.1098/rstb.2015.0113>

Accepted: 8 November 2015

One contribution of 16 to a theme issue 'Multifaceted origins of sex differences in the brain'.

### Subject Areas:

behaviour, genetics, health and disease and epidemiology, physiology

### Keywords:

sexual differentiation, sex differences, X chromosome, obesity, ischaemia, Klinefelter

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# The importance of having two X chromosomes

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Historically, it was thought that the number of X chromosomes plays little role in causing sex differences in traits. Recently, selected mouse models have been used increasingly to compare mice with the same type of gonad but with one versus two copies of the X chromosome. Study of these models demonstrates that mice with one X chromosome can be strikingly different from those with two X chromosomes, when the differences are not attributable to confounding group differences in gonadal hormones. The number of X chromosomes affects adiposity and metabolic disease, cardiovascular ischaemia/reperfusion injury and behaviour. The effects of X chromosome number are likely the result of inherent differences in expression of X genes that escape inactivation, and are therefore expressed from both X chromosomes in XX mice, resulting in a higher level of expression when two X chromosomes are present. The effects of X chromosome number contribute to sex differences in disease phenotypes, and may explain some features of X chromosome aneuploidies such as in Turner and Klinefelter syndromes.

## 1. Introduction

It comes as no surprise to us that males and females are different, because the differences are emphasized and celebrated in our daily conversations from the earliest years of our lives. In everyday discourse, biological sex differences are easily confused with gender differences, i.e. those stemming from cultural attitudes and sex-specific rearing. The study of *biological* sex differences attempts to identify, categorize and understand the *inherent factors* that make the two sexes different from each other. These include factors that make every female different from every male (and vice versa). In addition, some factors cause the two sexes to be different, on average, even though some individuals of each sex are similar to individuals of the other sex. Our general goal is to distinguish and understand the separate components causing sex differences.

At the beginning of life, in the zygote, all *inherent components* must be encoded on the X and Y sex chromosomes, because they are the only genetic factors that are different at that stage. The Y chromosome of mammals encodes several genes that eventually make males different from females, including the testis-determining gene *Sry* and genes required for spermatogenesis [1,2]. The action of *Sry* sets up lifelong differences in the levels of gonadal hormones, which act in each sex to make it different from the other sex. Until recently, the X chromosome was thought not to participate significantly in the process of sexual differentiation. That attitude probably stemmed partly from the idea that the process of X inactivation effectively silences most of one X chromosome in XX females, so that they, like XY males, have one active X chromosome in each cell. In the past decade, however, the study of mouse models has provided convincing evidence that cells with two X chromosomes are intrinsically different from those with one X chromosome. Sex differences caused by the number

of X chromosomes can have a profound effect on disease. A fundamental understanding of these diseases requires an appreciation of the effects of X chromosome number. The role of the X chromosome implies that specific X genes, at certain levels of expression, protect from disease and therefore might be novel targets for therapy.

## 2. Mechanisms causing sex differences because of the number of X chromosomes

The X chromosome is one of the most unusual chromosomes in mammals, because it is present in different numbers in males and females. There are numerous ramifications of this inherent imbalance. The inequality in genomic dose of X genes is thought to present a major problem [3], but perhaps for only some gene networks [4]. For some genes, having one or two doses does not make much difference, so that individuals with a single copy of the gene have about the same phenotype as those with two expressed copies. For other X genes, however, the level of expression must be within a limited range, over which the gene product has the optimal balance with its interacting partners, most of which are autosomal [5,6]. These X genes are called ‘dosage-sensitive’ genes. The existence of dosage-sensitive genes on the X chromosome means that cells with two X chromosomes will have too much of some gene products, and/or cells with one X chromosome will have too little, relative to their interacting partners in gene networks. This imbalance creates a selection pressure to increase the expression of the gene in XY cells, which leads to counteracting pressure to decrease expression in XX cells. These selection pressures are thought to have driven the evolution of the current dosage compensation system, X inactivation. The expression of X genes, from a single X chromosome in both sexes, is also upregulated by an unknown mechanism to make X gene expression about on a par with expression of autosomal genes, which are expressed from two copies in most instances [7]. X inactivation is a remarkable process that quite effectively reduces the expected  $XX > XY$  sex difference in expression of all but a small minority of X genes [8].

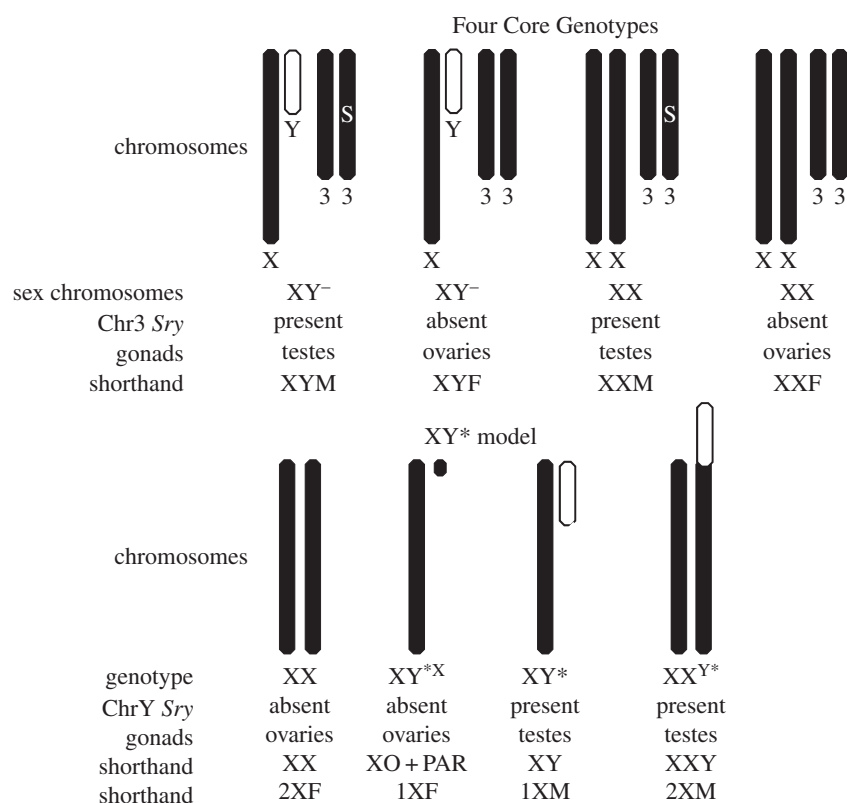
Because X inactivation is a random process in somatic tissues derived from the embryonic epiblast, each XX cell expresses most gene variants and parental imprints from only one of the two X chromosomes. Adult XX tissues and individuals are therefore mosaics of cells that exhibit the effects of either the maternal or paternal X genes. No such mosaicism occurs in XY tissues. The mosaicism of X gene effects has long been recognized as one of the factors that makes individuals with two X chromosomes different from those with one [9,10]. In general, mosaicism is viewed as protective against disease, thus benefitting females. If a deleterious X mutation (or imprint) is inherited from the mother, that mutation is expressed in all cells of XY individuals, because of the male’s hemizygous exposure of X alleles, but in only about half of the cells of XX (or XXY) individuals. Thus, X-linked mutations affect males more than females (e.g. as in X-linked developmental disabilities such as Fragile X syndrome). More generally, any genetic variation among X alleles, even those not causing overt disease (e.g. red-green colour blindness), will cause sex differences in traits because the effect of the variant is mitigated in XX tissues by the presence of another variant, but not in XY tissues. The ‘mosaicism

buffering effect’ that causes sex differences is relevant only to genetically diverse populations such as humans, but is not a potential explanation of sex differences in traits in inbred laboratory populations such as inbred mice in which the maternal and paternal X alleles are identical.

The sexual inequality of number of X chromosomes also leads to sex differences in traits via at least three mechanisms other than mosaicism: escape from X-inactivation, X imprinting and epigenetic sinks [11]. (i) *Escape from X inactivation*. X inactivation is not 100% complete, because some genes are insulated from the inactivation process and are thus expressed from both X chromosomes, making expression levels inherently greater in XX (and XXY) cells than in XY (or XO) tissues. The number of ‘X escapees’ has been estimated at about 15% of X genes in humans, and 3% in mice [12–15]. These estimates are based on studies of cell lines under artificial conditions *in vitro*, in which it is possible to detect rigorously even small amounts of expression from the inactive X chromosome. Evidence suggests, however, that many of the putative X escapees do not show the expected  $XX > XY$  pattern of expression in whole tissues *in vivo* [16,17], and the degree of escape from inactivation might be specific to cell types, developmental stages, disease states, environmental conditions, etc. [18]. (ii) *Parental imprinting*. During production of gametes, each parent methylates DNA in some genes, silencing the allele passed from that parent to its offspring, a process known as imprinting. Sex differences in traits might also arise because of the inherently different pattern of parental imprinting of the X chromosome. Unlike XY tissues, XX tissues are influenced by parent-of-origin effect on X genes. Each parental imprint affects expression in about one-half of the cells because of random X inactivation. For example, a paternal imprint affects only XX cells in which the paternal X chromosome is active. (iii) *Epigenetic sinks*. The presence of a large inactive and heterochromatic X chromosome in XX cells may attract heterochromatizing factors away from other chromosomes (providing a ‘sink’ for those factors) which could shift the epigenetic status of the genome, and shift gene expression. The ‘epigenetic sink’ hypothesis is still speculative, but has some support [19–23].

## 3. Methods for detecting differential effects of two versus one X chromosome

Our goal is to test for phenotypic effects of the number of X chromosomes, mirroring the natural difference between females and males, in a manner that will reveal X gene effects involving the molecular mechanisms outlined in §2. We note that some traditional methods of linking genes to phenotypes may not uncover these kinds of X chromosome dosage effects. For example, traditional linkage or association analyses, which establish that variations in the genomic sequence cause phenotypic variation, do not test directly for effects of different doses of genes when there is no difference in genomic DNA sequence. Although variations in genomic sequence might cause changes in gene expression that accidentally mimic sex differences in levels of expression, they do not necessarily do that, and X escapees may have no endogenous differences in DNA sequence. Moreover, many linkage and association studies do not include the X chromosome because of the complexity of analysis of that



**Figure 1.** Effects of one versus two X chromosomes can be revealed using two mouse models that have various combinations of sex chromosomes and gonad type. In the FCG model, the Y chromosome is deleted for *Sry*, and designated  $Y^-$ . An *Sry* transgene (S) is present on chromosome 3 in some groups. Breeding  $XYM$  with  $XXF$  produces the four genotypes,  $XX$  and  $XY$  mice with testes ( $XXM$ ,  $XYM$ ), and  $XX$  and  $XY$  mice with ovaries ( $XXF$ ,  $XYF$ ). In the  $XY^*$  model, breeding an  $XX$  mother with  $XY^*$  father produces the four genotypes, based on the abnormal recombination of the  $Y^*$  chromosome with the X chromosome [43–45]. Adapted from [27] with permission from Elsevier.

chromosome [24,25]. However, methods that vary the copy number of the X chromosome to observe its effects *in vivo* have been informative [25].

We discuss here mouse models for comparing mice with different numbers of the X chromosome [26–28], because this species is the most genetically tractable whole-animal mammalian model of human physiology and disease. An important problem is that groups with different numbers of X chromosomes could conceivably have different levels of gonadal hormones, so that group differences might be caused by gonadal hormones rather than direct effects of X genes on non-gonadal tissues. For example, naturally occurring variation in the number of X chromosomes in humans is associated with changes in adult gonadal hormone levels. Women with Turner syndrome (XO) are infertile and have altered levels of androgens and oestrogens, compared with XX females [29]. Klinefelter syndrome (KS) men (XXY) are also infertile and have lower levels of androgens than XY men [30]. XXY mice similarly are infertile and have lower levels of androgens compared with XY [31–33], but XY and XXY male mice appear to have similar levels of androgens prenatally [34]. The endocrine differences between XO and XX mice are reduced relative to those in humans. XO mice are fertile in some genetic backgrounds, and prenatal levels of androgens appear to be similar to those of XX mice [34]. Nevertheless, one cannot assume that there are no differences in the levels of ovarian hormones. Accordingly, methods must be used to deal with the possible problem that varying X chromosome number could bring changes in the levels of gonadal hormones, which cause differences in non-gonadal

traits. Distinguishing hormonal and non-hormonal effects of X chromosome number is a challenge.

One approach to circumventing the issue of confounding gonadal hormone levels is to gonadectomize (GDX) mice as adults (with or without equal hormone replacement) to control hormone levels, so that groups of adult mice can be effectively compared when hormonal levels are the same [35]. Although that method eliminates many possible confounding effects of hormones, it is not sufficient to eliminate all conceivable group differences caused by gonadal hormones. Gonadal hormone effects can be long-lasting, so that group differences may exist even before birth, or before the time of GDX, and can potentially cause phenotypic differences in adulthood [36,37]. (Nevertheless, prenatal differences in gonadal hormone levels, in mice with the same type of gonad, have not been detected in two mouse models discussed here, the Four Core Genotypes (FCG) and  $XY^*$  models [34,38].) Ultimately, there are almost no methods for keeping gonadal hormones equivalent among groups during the entire lifetime, except in mice that lack gonads entirely [39–41]. However, as we see in §4, it is possible to discover differences in mice with different sex chromosomes that are not explained by effects on gonadal secretions.

One useful model is the FCG model [28,35,42], which produces  $XX$  and  $XY$  mice with testes ( $XXM$  and  $XYM$ ) or with ovaries ( $XXF$  and  $XYF$ ) (figure 1). Thus, the  $XX$  versus  $XY$  comparison can be made when both groups have testes, or have ovaries. The  $XX$  and  $XY$  groups with the same type of gonad have similar levels of gonadal hormones in adulthood [46–50]. Moreover, differences between  $XX$  and  $XY$  mice are

found after the gonads are removed in adulthood (making their adult gonadal hormone levels zero, and equivalent across groups). In some cases, the XX versus XY difference observed in mice with ovaries is similar to the XX versus XY difference observed in mice with testes. That result suggests that sex chromosome differences can occur under quite different hormonal conditions including during prenatal life (see examples in §4). The FCG model has the major advantage that it detects XX versus XY differences that are independent of type of gonad. The model does not solve whether these sex chromosome effects are caused by the number of X chromosomes (1 versus 2) or the presence of the Y chromosome. For that purpose, the XY\* model is useful. It compares groups that have sex chromosomes that are similar to XO, XX, XY and XXY (figure 1). In this model, two comparisons test for different effects of one versus two X chromosomes: XO versus XX, and XY versus XXY (figure 1). The combined use of the FCG and XY\* models offers the advantage that a sex chromosome effect can be detected and found to be insensitive to gonadal type in the FCG model, and then confirmed in a completely different genetic model using XY\* mice, which also discriminates between effects caused by X or Y chromosome number. The XY\* model may be used by itself, to demonstrate differences in the effects of one versus two X chromosomes, in the presence (XY versus XXY) or absence (XO versus XX) of the Y chromosome [51]. The XY\* model also tests for the effect of a Y chromosome (XO versus XY or XX versus XXY), but in this model the Y chromosome effects are most likely the result of effects of testicular secretions, because the mice with Y chromosomes have testes. In §4(a–c), we illustrate the use of these and related mouse models to identify mechanisms that contribute to sex differences in traits such as obesity, cardiovascular disease and behaviour.

## 4. Test cases showing effects of X chromosome number

### (a) Sex differences in metabolism and adiposity

Women and men differ in the amount and distribution of fat in the body, and overweight and obesity have differential effects on health of the two sexes [52]. Mice are an important genetic model in research on obesity and metabolic syndrome. As adults, male mice generally weigh more than female mice. In the C57BL/6 strain, the sex difference is approximately 25%. This difference can be seen in gonad-intact FCG mice (figure 2a). By 45 days of age, after puberty, XXM or XYM mice (males with testes) weigh more than XXF and XYF (females with ovaries). To test if the sex difference is caused by adult secretions of gonadal hormones, the gonads are removed (figure 2b). Within a month after GDX, all four groups weigh about the same, because of reduced increase of body weight in males and increase in body weight in females, indicating that the main sex difference in body weight was influenced by both testicular and ovarian secretions [43,53,55]. In mice gonadectomized for seven weeks or longer, however, the XX mice gradually gain weight relative to XY mice, leading to a large  $XX > XY$  difference in body weight by eight months after GDX. This demonstrates that two X chromosomes are associated with enhanced body weight, a finding that could never be determined by comparing

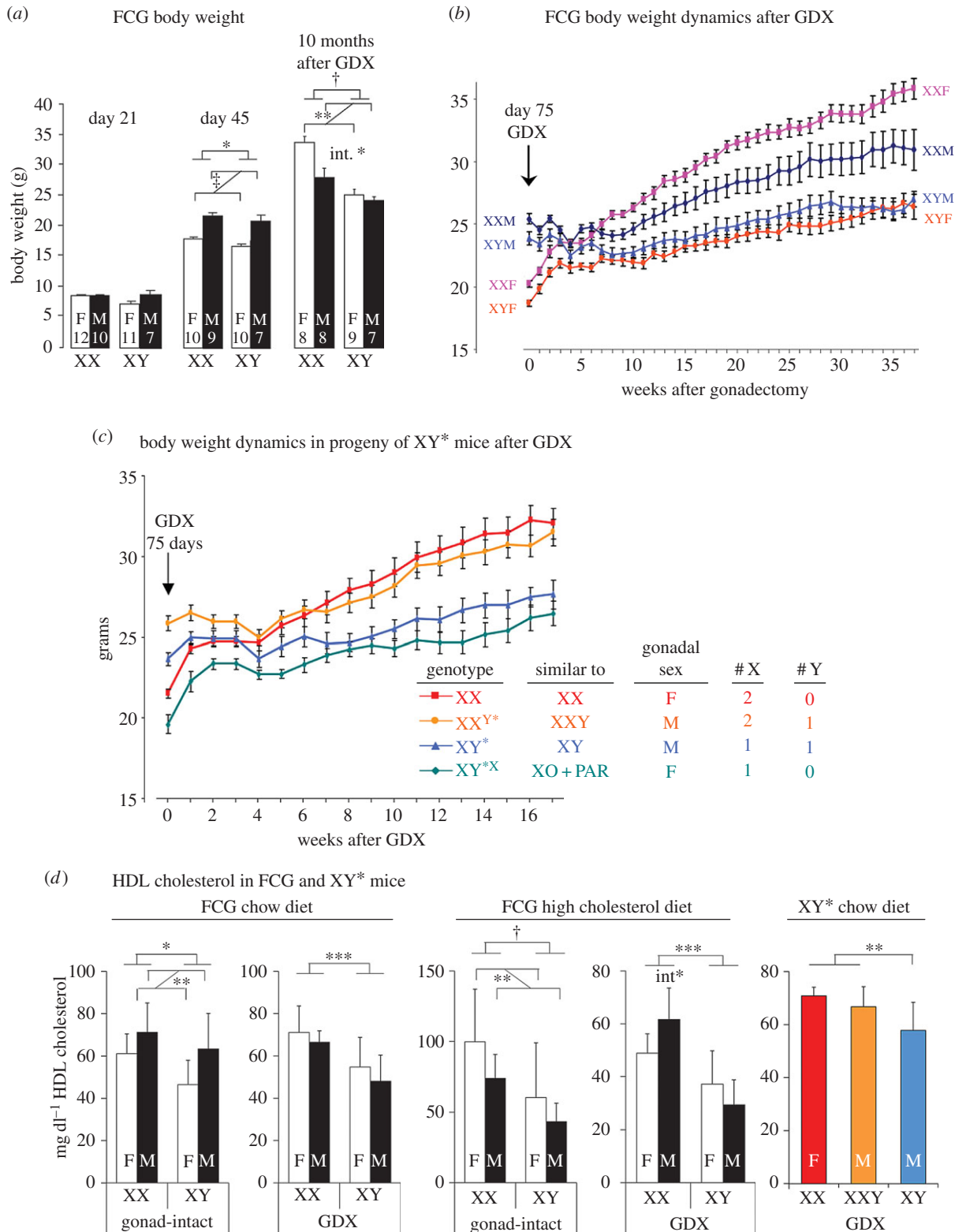
standard XX female and XY male mice, which differ from one another in both gonad type and sex chromosome complement. This sex chromosome effect cannot be explained by group differences in gonadal hormones secreted in adulthood, because the groups had no gonadal hormones for a prolonged period of adulthood. The effect is also not likely to have been caused by group differences in the levels of gonadal hormones secreted before GDX in adulthood, because the  $XX > XY$  difference occurs when comparing XX and XY groups that both had either testes or ovaries. The sex chromosome effect therefore occurs robustly under distinctly different hormonal conditions. Moreover, comparison of XX and XY FCG mice with the same type of gonad has uncovered no prenatal or adult  $XX - XY$  difference in levels of gonadal hormones [38,46–50].

The XX chromosome effect on body weight could potentially be caused by the presence of two X chromosomes, or by the absence of a Y chromosome. To distinguish these possibilities, mice from the XY\* model were studied. In mice gonadectomized as adults, body weight increased in mice with two X chromosomes (XX and XXY) more than in mice with one X chromosome (XO or XY), but the presence or absence of the Y chromosome had little apparent effect (figure 2c) [53]. Thus, the effect is caused by the inherent sex difference in number of X chromosomes. The results from XY\* mice confirm and extend those from FCG mice, using a different genetic model.

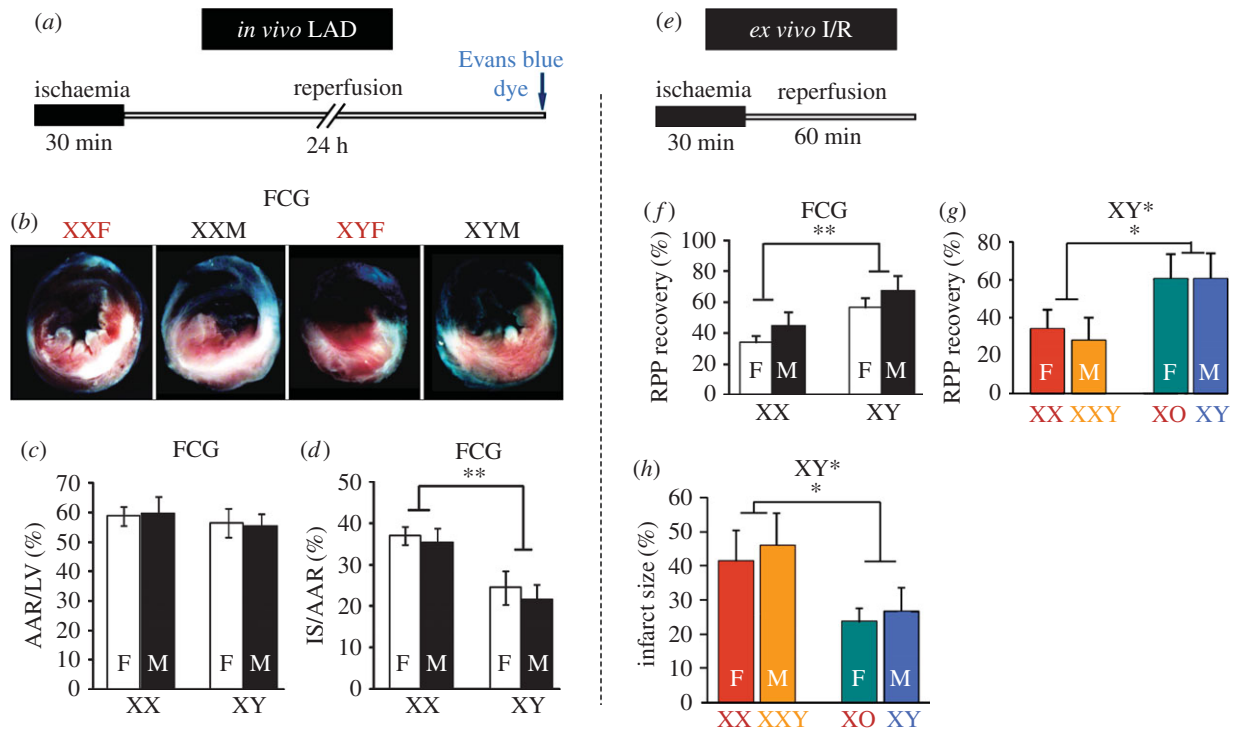
The X chromosome effect on body weight is associated with several metabolic changes. One reason for the greater body weight and adiposity of XX mice, relative to XY, could be that they begin their diurnal phase of feeding earlier, and ingest more food during the light phase of the cycle [53,55]. Thus, the time of food intake, rather than total amount eaten, could be affected by the number of X chromosomes. Mice with two X chromosomes also have higher expression of growth hormone mRNA in the arcuate nucleus of the hypothalamus, which likely reflects different activity of hypothalamic circuits regulating feeding [56,57]. Mice with two X chromosomes (relative to those with one) also express higher levels of the gene *Pdyn* in the striatum [58], which is conceivably related to feeding behaviour [59]. The changes in feeding in XX mice gonadectomized as adults likely contribute to the accrual of nearly double the levels of body fat as XY mice [53]. Moreover, when GDX XX mice are fed a high fat, simple carbohydrate diet, they gain weight faster than XY mice, develop insulin resistance, and have greatly elevated levels of liver fat [53]. In addition, XX mice have altered levels of circulating lipoproteins compared with XY mice, including about 20% higher levels of high-density lipoproteins, both when they are gonad-intact and after GDX, and when fed different diets (figure 2d) [54]. Again, this difference is attributable to the number of X chromosomes.

### (b) Sex differences in cardiovascular disease

Men and women differ in their susceptibility to cardiovascular artery disease, which is a leading cause of death [60], but the biological basis of the sex difference is not well understood. The FCG and XY\* mouse models have also been studied in a mouse model of ischaemia/reperfusion (I/R) injury in the heart [61] (figure 3) modelling a human heart attack. FCG and XY\* mice were GDX and used one month later to remove any possible group differences in levels of gonadal hormones. In an *in vivo* model, blood flow to some regions of the heart is stopped by ligating one of the cardiac arteries for



**Figure 2.** The FCG and XY\* models are used to demonstrate that the number of X chromosomes contributes to sex differences in body weight and lipoprotein levels. (a) At weaning (age 21 days), the FCG groups have similar body weight, but after puberty (day 45), mice with testes had greater body weight than mice with ovaries ( $\dagger p < 0.000001$ ), and mice with XX sex chromosomes weighed slightly more than XY ( $*p < 0.05$ ). After mice were gonadectomized (GDX) at 75 days of age, and allowed to grow for 10 months, XX mice weighed much more than XY mice, in both gonadal male groups and gonadal female groups ( $\dagger p < 0.0001$ ), and females were heavier than males ( $**p < 0.01$ ). The effects of sex chromosome complement interacted significantly with the effects of gonad type (int,  $*p < 0.05$ ). (b) Growth curves for FCG mice GDX at day 75 (week 0). Before gonadectomy (GDX), mice with ovaries weighed less than mice with testes, and XX mice weighed more than XY mice. The sex difference caused by gonadal secretions acting in adulthood disappeared within four to five weeks after GDX, and thereafter XX mice gained more weight than XY mice. (c) The sex chromosome effect on body weight is confirmed in the XY\* model and found to be caused by the number of X chromosomes, using the same GDX design as in a and b. After GDX at day 75, mice with two X chromosomes gained weight more than mice with one X chromosome ( $p < 0.000001$ ). (d) Metabolic effects of X chromosome number. In FCG mice (left and centre panels) that were gonad-intact or GDX, and in mice eating low-fat lab chow or a high cholesterol diet, XX mice had consistently higher plasma levels of high-density lipoprotein (HDL) cholesterol, independent of their gonadal sex. In the XY\* model, mice with two X chromosome had higher HDL levels than mice with one X chromosome.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $\dagger p < 0.0001$ . Adapted from [53,54], with permission from Wolters Kluwer Health, Inc.



**Figure 3.** Use of the FCG model shows that after ischaemia/reperfusion injury, GDX XX mice have worse recovery and larger myocardial infarct area compared with GDX XY mice, irrespective of gonadal type. (a) Experimental protocol *in vivo*: the left anterior descending artery was occluded in GDX FCG mice for 30 min followed by 24 h of reperfusion. (b) Representative cross sections of heart muscle stained with triphenyl tetrazolium chloride. The white area represents the infarcted area, blue shows the non-infarcted area, red plus white areas show risk area. (c) Percentage of area at risk (AAR) divided by left ventricle area. (d) Infarct size (IS) divided by AAR.  $**p < 0.01$ ,  $n = 6-7$ . (e) Experimental protocol *ex vivo*: perfusion of the heart is shut off for 30 min, then reperused for 60 min before measuring heart function. (f) The rate pressure product (RPP), a measure of recovery after injury, was worse in XX than XY mice.  $**p < 0.01$ . (g) Use of the XY\* model shows that in the *ex vivo* system, mice with two X chromosomes (XX, XXY) have worse recovery (lower RPP) than mice with one X chromosome (XO, XY). (h) The infarct size as the percentage of total ventricular area in hearts *ex vivo*.  $*p < 0.05$ . Adapted from [61] with permission from Oxford University Press.

30 min, then the ligature is removed to start the blood flow to the heart muscle for 24 h, producing I/R injury (figure 3a-d). In an *ex vivo* model, in which the heart is removed from the mouse and perfused through the aorta with an oxygenated physiological buffer, the flow is interrupted for 30 min and then restarted for 60 min to simulate I/R injury in humans (figure 3e-h). In both *in vivo* and *ex vivo* models, the infarct size was measured at the end of the experiment. In the *ex vivo* model, haemodynamic parameters were measured throughout the experiment such as rate pressure product (RPP). The infarct size in GDX FCG mice was strikingly greater in XX than XY (approx. 40%) in the *in vivo* model, independent of the gonadal sex in mice (figure 3b-d). Consistent with larger infarct size in XX mice *in vivo*, the heart functional recovery after ischaemia in the *ex vivo* model was significantly lower in XX than XY mice in GDX FCG mice, as indicated by RPP (figure 3f). When the XY\* model was subjected to *ex vivo* I/R injury, RPP was significantly lower and the infarct size was significantly larger in mice with two X chromosomes, relative to mice with one X chromosome, and the presence of the Y chromosome appeared to have no effect (figure 3g,h). These studies of X chromosome effects on cardiovascular disease mirror the studies on metabolism discussed in §4(a), in that two X chromosomes confer a greater disease burden than one X chromosome.

### (c) Sex differences in behaviour

Studies of mouse behaviour also provide evidence that the number of X chromosomes contributes to sex differences.

Fear reactivity in gonad-intact adult mice is greater in XO than XX mice, shown by the reluctance of the mouse to venture into an open area of an elevated plus maze. This difference is not explained by the number of genes in the pseudoautosomal region (PAR), but by the difference in number of non-PAR X genes [62]. In studies of sexual behaviour, when mice of the XY\* model are GDX as adults and treated equally with testosterone, then tested with a receptive female, they show male sex behaviour differently depending on the number of X chromosomes [34]. In juvenile mice of the XY\* model tested at approximately three weeks of age, mice with one X chromosome show less social behaviour when paired with another mouse, compared with mice with two X chromosomes [51]. Mice with one X chromosome investigated their cage partners more frequently than mice with two X chromosomes, but spent less total time in proximity to or interacting with the partner. When tested for preference for a novel versus familiar mouse, mice with two X chromosomes had greater preference for the unfamiliar mouse. The greater anxiety-like behaviour, found in adult mice with one X chromosome [62], was also found in juvenile mice [51], and may help explain the tendency of mice with one X chromosome to avoid novel mice or social partners, more than mice with two X chromosomes.

### (d) Models of sex chromosome aneuploidy

The effects of two versus one X chromosome are not only relevant to the natural difference between the sexes (XX versus XY), but also to two sex chromosome aneuploidies



that occur with significant frequency in humans: XO (Turner syndrome, 1/2500 live female births) and XXY (KS, 1/600 live male births). XO females differ from XX females exclusively because of the difference in number of X chromosomes, and XXY males differ from normal XY males for the same reason. XXY males experience inactivation of one of the two X chromosomes, as in XX females [63]. Monosomy of the X chromosome (XO) is usually lethal to human embryos [64], but those who survive to adulthood have multiple phenotypes including ovarian failure with abnormal levels of reproductive hormones (low oestrogen and elevated androgens), short stature, neck webbing, and susceptibility to cardiovascular and metabolic disease [29]. Men with KS have small testes, lowered testosterone levels, and increased height. As a group they show greater incidence of behavioural problems including delayed language development and deficits in social and executive functioning [65]. They experience increased prevalence of several health problems [66,67], some of which are usually more common in women than in men including breast cancer [68,69], osteoporosis [70] and autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosus [71]. KS men have increased body fat, specifically abdominal fat, as well as increased rates of hyperinsulinaemia, insulin resistance, type II diabetes and metabolic syndrome [30].

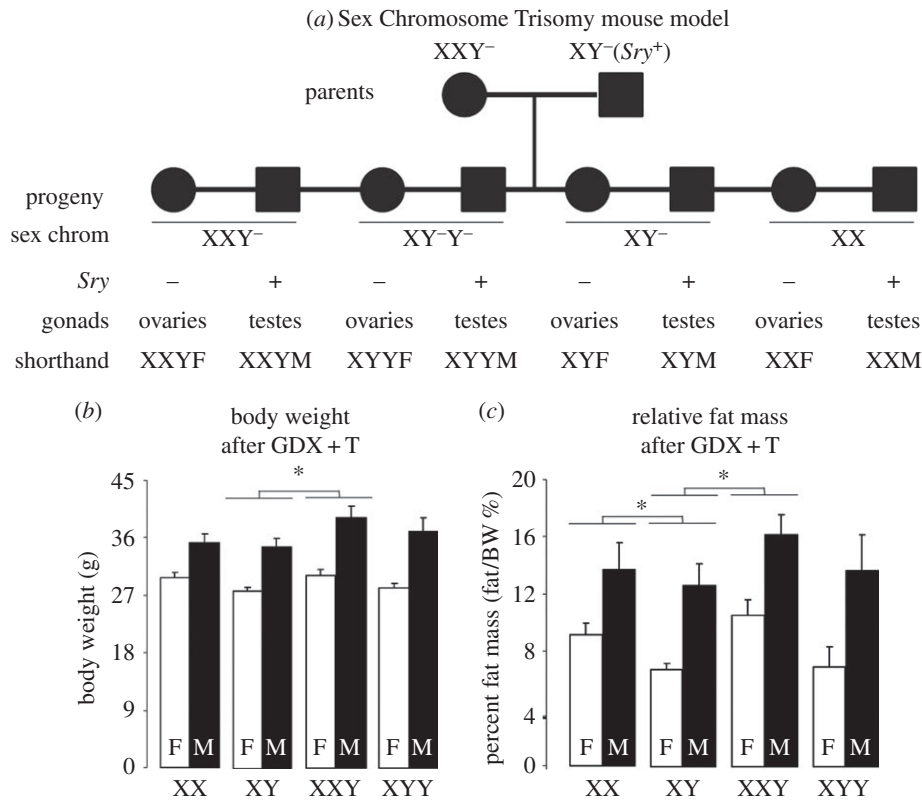
The loss of one X chromosome in female mice results in a much milder phenotype than in humans. XO mice are fertile at least in outbred genetic backgrounds. The milder phenotype might result from a smaller number of genes in the mouse PAR than human PAR, which are expected to be expressed differently in XO (one PAR) versus XX (two PARs). In addition, fewer X genes escape inactivation in mice than humans, which would reduce the presumed disparity of expression levels of non-PAR X escapees [72], so fewer phenotypes would be affected. Differences in gene expression have been detected in XO mice relative to XX, including in X escapees [73,74]. Although XO mice do not completely model Turner syndrome, numerous genes escape X inactivation in both species, so that the XX versus XO comparison in mice may well model some effects of dosage of those genes in humans.

Several mouse models of KS have been used [33,75–77]. These studies have demonstrated that the second X chromosome in males eliminates sperm production as in KS men [78], reduces testis size, lowers testosterone levels, induces Leydig cell hyperplasia and causes behavioural deficits [33,79]. Moreover, XXY mice have abnormal bone density, and altered sexual partner preferences [80,81]. In most of these studies, gonad-intact mice were studied, so that the lower levels of testosterone in XXY mice, relative to XY mice, could have caused the difference in phenotype, instead of direct (non-gonadal) effects of X genes in mice with one versus two X chromosomes. In some cases, the group differences in adult levels of testosterone were eliminated by castration of adult males, with or without replacement of testosterone [34,80–82]. For some behaviour traits, such as altered social interaction and sex preference, equalizing hormone levels ameliorated group differences [81]. However, differences in male sexual behaviour and in bone architecture persisted despite castration and testosterone replacement [34,80]. These studies ruled out effects of circulating testosterone as the responsible mechanism, but in these studies it is difficult to assess if XY versus XXY differences in levels of testicular hormones before the time of GDX contributed to group differences in phenotype.

Recently, we introduced a novel model of KS, the ‘Sex Chromosome Trisomy’ (SCT) model, which produces XX, XY, XXY and XYY mice (figure 4a) [77,83]. In this model, as in the FCG model, the *Sry* gene is not on the Y chromosome, but is present on an autosome as a transgene. Thus, each of the four sex chromosome groups is produced with *Sry* (with testes) or without *Sry* (with ovaries). The comparison of XXY groups, modelling KS versus normal men, tests for the effects of one versus two X chromosomes when a Y chromosome is present. The model expands the ability to detect effects of the second X chromosome that do not depend on testicular secretions, because the model tests the effects also in mice that do not have testes. In SCT mice GDX as adults and then treated equally with testosterone, XXY mice weigh more than XY mice and have more body fat and less lean mass, compared with XY mice (figure 4b) [77]. The occurrence of these differences in mice that had either testes or ovaries indicates that the group differences do not depend on XXY versus XY differences in the levels of testicular hormones. In tests of sexual partner preference of SCT mice, again GDX and treated with testosterone, XXY male mice also spent less time with a female test mouse than did XY male mice, suggesting a feminizing influence of the second X chromosome on sexual partner preference [83]. This influence appeared to be dependent on the presence of the Y, as the partner preference of XX male mice did not differ from XY males. In the same mice, gene expression was measured in the bed nucleus of the stria terminalis and the striatum. Most genes in XXY males were found to be male-typical in their expression patterns, but a substantial minority of genes in both regions was significantly more female-typical. These genes are candidates for genetic contributors to the KS phenotype

## 5. Candidate X genes that cause sex differences

The inherent sexual imbalance in the number of X chromosomes has now been shown to have unexpectedly large effects on phenotypes, including susceptibility to disease. The next step is to identify the X genes that contribute to this imbalance, and to understand the downstream pathways that affect phenotypes. Although the X chromosome is large and gene-rich, the candidate genes are less numerous, because we can focus on either imprinted genes or genes escaping X inactivation. Tissue-specific parent-of-origin effects on gene expression are just now being reported for an increasing number of X genes [84–86], but it is not clear how many of these will emerge as viable candidates for explaining phenotypic sex differences caused by different number of X chromosomes. By contrast, the number of X escapees resulting in sex differences in expression is relatively small [13], making their analysis more tractable and attractive at present. In mice, several X escapee genes are particularly interesting: *Kdm5c*, *Kdm6a*, *Eif2s3x* and *Ddx3x*. These are routinely found by numerous laboratories to be expressed at higher levels in XX (or XXY) than XY (or XO) mice in numerous tissues [53,56,61,73,74,87–92]. Each escapes X chromosome inactivation in humans and mice [93–97]. Two of these genes, *Kdm5c* and *Kdm6a*, are histone demethylases that are expected to have widespread effects on gene expression throughout the genome, and null mutations of each are implicated in human disease [98,99]. *Ddx3x* is an RNA helicase involved in several basic cellular processes such as transcription, RNA transport



**Figure 4.** The Sex Chromosome Trisomy model. (a) The model involves crossing FCG XY<sup>-</sup>(Sry<sup>+</sup>) male (same as XYM in figure 1) with XXY<sup>-</sup> female who has the same Y<sup>-</sup> as in the FCG model (figure 1). The father's Sry is transgenic on chromosome 3. Eight genotypes are produced, XX, XY, XXY and XYY, each with either testes or ovaries. (b) Body weight data from SCT mice shows that after gonadectomy (GDX) in adulthood and treatment with testosterone (T), XXY mice weigh more (b) and have more body fat relative to body weight (c), compared with XY mice. Adapted from [77,83].

and splicing, and translation, and is implicated in human cancer and intellectual disability [100]. Less is known about *Eif2s3x*, a translation initiation factor, which is the X-linked paralogue of *Eif2s3y*, a spermatogonial proliferation factor [101]. Each of these genes has a closely related gene on the Y chromosome, the expression of which in XY males could compensate for the lack of a second copy of the X gene in males. However, the Y genes are unlikely to offset sex differences in effects of X escapees. In some cases, the X and Y copies appear to have diverged in function [102] or pattern of expression [89,90], suggesting that the compensation by the Y paralogue is not complete. Thus, the X escapee genes remain exciting candidates for explaining the effects of two X chromosomes relative to one. Tests of this idea require careful manipulation of the dose of the genes in mouse models, to determine if one versus two copies of the gene causes phenotypic changes similar to the comparison of one versus two copies of the entire X chromosome.

## 6. Conclusion and prospectus

In the twentieth century, gonadal hormones emerged as the primary proximate factors that act on tissues to cause sex

differences in phenotypes. Their effect was so pervasive that they were essentially the only proximate factors incorporated into theories of the origins of sex differences in phenotype. In the past two decades, however, the sexual imbalance of effects of the X and Y chromosomes have been clearly shown to cause sex differences in non-gonadal tissues that are not mediated by gonadal hormones [10,11,35,103–105]. More of these effects have been localized to the X chromosome than to the Y chromosome. Although specific X genes are prime candidates for these effects, we cannot rule out non-genetic effects of the X chromosome as reviewed above. These studies are still in their infancy, because we do not know yet which X genes are responsible, and how they act. An important question that is almost completely unstudied is how the multiple sex-biasing factors, hormones and sex chromosome genes, interact with each other in specific instances. Thus, we can expect exciting developments in the near future.

**Authors' contributions.** A.P.A. drafted the manuscript, which was edited by all other authors.

**Competing interests.** The authors declare no competing interests.

**Funding.** Supported by DK083561 (A.P.A., X.C., K.R.), HL119886 (M.E., A.P.A.), HD076125 (A.P.A., E.V.), HL90553 (K.R.), NS043196 (A.P.A.), T32GM007185 (J.C.L.) and T32HD007228 (S.M.W.).

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