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Sex-specific adaptation and genomic responses to Y chromosome presence in female reproductive and neural tissues

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Y chromosomes typically harbour a small number of genes and an abundance of repetitive sequences. In *Drosophila*, the Y chromosome comprises multimegabase long segments of repetitive DNA and a handful of protein-coding genes. In mammals, the Y chromosome also harbours a disproportionally high abundance of repeats. Here, we built on a *Drosophila melanogaster* model in which the Y chromosome is decoupled from sexual determination. Genotypes were genetically identical for the autosomes, X chromosome, and mitochondria, but differ by the presence or dose of the Y chromosome. Addition of an extra Y chromosome had limited impact in males. However, the presence of a Y chromosome in females induced a disproportionate response in genes expressed in the ovaries as well as genes encoded by the mitochondrial genome. Furthermore, the data revealed significant consequences of Y chromosome presence in larvae neuronal tissue. This included the repression of genes implicated in reproductive behaviour, courtship, mating and synaptic function. Our findings exhibit the Y chromosome as a hotspot for sex-specific adaptation. They suggest roles for natural selection on Y-linked genetic elements exerting impact on sex-specific tissues as well as somatic tissues shared by males and females.

1. Introduction

Y chromosomes present a number of molecular features that evolved in part due to their experience of higher mutation rates, restriction to male lineages, stronger selective pressures for male fitness and inability to purge individual mutations due to the lack of recombination. Thus, the Y chromosome is repeat rich in most organisms, with multicopy genes, repetitive arrays and disproportionate amounts of satellite DNA and transposable elements (TEs). The *Drosophila melanogaster* Y chromosome is an extreme case: it harbours multimegabase long stretches of satellite DNA repeats and an exceptionally high density of TEs [1–3]. Based on the gene density of X-linked euchromatic segments, over 4000 genes were expected in this approximately 40 Mb Y-linked DNA segment that accounts for about 20% of the male haploid genome. However, the chromosome only contains some 14 protein-coding genes, many of which appear to be recently acquired through transposition from autosomal and X-linked genes [4].

Males and females have distinct genetic needs for optimal fitness, with asymmetrically transmitted genetic elements serving key roles in sex-specific adaptation [5–13]. While the Y chromosome accumulates evolutionary adaptations that favour males, sex linkage also removes selective costs due to male–female trade-offs [14,15]. The Y chromosome is expected to evolve buffering mechanisms to counter deleterious outcomes emerging from female-selected genetic elements. This includes, for instance, costs emerging from variation in mitochondrial DNA (mtDNA), a genome that accumulates evolutionary variation with disproportionate consequences to spermatogenesis and male fertility [12,16–18]. Finally, the *Drosophila* sex chromosomes are sensitive to epigenetic

modifications that occur along spermatogenesis or oogenesis [19–23], which add another layer of complexity in their regulatory interactions.

In mammals, the presence of the Y chromosome in females has been detected across several brain regions [24], and its abundance increases during pregnancy [25]. The event is supported by stable incorporation of DNA injected intravenously into mice [26] and experimental tests revealing that male fetal stem cells can colonize maternal tissues and differentiate into tissue-specific resident cells, including neurons [27,28]. Despite suggestions that sex chromosomes play a special role encoding sex-differences in brain function [29–31], the physiological and functional significance of Y-linked DNA in male or female tissues have remained unclear [26,27,32–34].

To investigate the impact of a male-restricted DNA on regulatory outcomes, we constructed *Drosophila* strains that differ from their progenitor only in the presence/dose of the Y chromosome but were otherwise genetically identical for all other chromosomes and mitochondria. Surprisingly, the presence of an extra Y chromosome in *XY/Y* male genotypes had limited impact on global gene expression levels. Our observations indicated that a female-restricted tissue (ovary of adult flies) was disproportionally sensitive to Y chromosome presence. Nevertheless, Y chromosome presence on the central nervous system (CNS) of third instar larvae, a somatic tissue common to males and females, was also significant. The data showed that the presence of a male-adapted chromosome in larval neuronal tissues significantly influenced genetic pathways associated with reproductive behaviour, mating and synaptic function. These changes were accompanied by evidence of genetic interaction between the Y chromosome and the mitochondria.

2. Material and methods

(a) Introgression of the Y chromosome into isogenic backgrounds

Y chromosome substitution lines were produced as previously described [9] (electronic supplementary material, figure S1), with the *4361* strain subjected to 24 generations of brother–sister mating. Y chromosome substitution strains, denoted as *4361(c)* and *4361(o)*, are genetically identical except for the origin of the Y chromosome. Next, we generated isogenic strains with an extra Y chromosome. The key strain in this procedure is a *C(1;Y)3, In(1)FM7, w*[1] *m*[2]/0/*C(1)M4, y*[2] genotype (Bloomington stock number 995) [35]. Note that *C(1;Y)3, In(1)FM7, w*[1] *m*[2]/0/*C(1)M4, y*[2] does not have a free Y chromosome (hereafter denoted *X^X/0/X^Y*). Both attached *X^X* and *X^Y* chromosomes are remarkably stable in the *X^X/0/X^Y* configuration [36], and chromosome breaks are readily visualized. The *X^X/0/X^Y* lineage was subjected to five generations of inbreeding through brother–sister mating immediately prior to the start of the study. Genotypes that differ only by the presence of the Y chromosome (*X^X/0* and *X^X/Y*) and the number of Y chromosomes (*X^Y/0* and *X^Y/Y*) were produced and phenotypically monitored (electronic supplementary material, figures S2 and S3). The presence and dose of the Y chromosome in *X^X/Y* and *X^Y/Y* were validated by PCR of the Y-linked gene *kl-3*. The strains (*X^Y/Y* and *X^X/Y*) were designed to be genetically identical to the progenitor strain of the same sex (*X^Y/0* and *X^X/0*), except for Y chromosome number and origin. Genotypes [*995*, *995(c)* and *995(o)*] were sequenced with shotgun Illumina sequencing. RNA-seq of whole flies was performed in replicated pools of approximately 30 virgin flies of the same sex and aged for 48 h post-eclosion.

(b) Ovary and testis dissection

Virgin males and females 48 h post-eclosion were collected. Testes and ovaries were dissected and isolated [37,38]. The corresponding carcasses of females were saved to confirm the introgression of the Y chromosome by PCR of a 180 bp fragment from the third exon of the Y-linked gene *kl-3*. After confirming the presence of the Y chromosome, total RNA was isolated from two biological replicates with 10 pairs of ovaries in each, and submitted for RNA-seq.

(c) Larval central nervous system dissection and genotyping

The CNS was isolated according to Egger *et al*. [39]. Briefly, L3 larvae were dissected in 20 µl of phosphate-buffered saline under a stereomicroscope. The clean CNS was individually immersed in 20 µl of ice-cold TRIzol, flash frozen and stored at −80°C [39]. Matched carcasses were saved to confirm the genotype of the sample (electronic supplementary material, figure S4). RNA from each carcass was isolated with 200 µl of TRIzol and Direct-zol RNA miniprep columns (Zymo Research). RNA was eluted with 30 µl of nuclease free water and checked with Nanodrop (Thermo Fisher Scientific). The sex of larvae was determined with PCR of the eighth exon of the Y-linked gene *kl-3* or the control *eif5*, and validated along positive (pool of adult male flies) and negative (pool of adult female flies) controls (electronic supplementary material, figure S5*a*). The presence of the Y chromosome was confirmed by PCR of the Y-linked gene *kl-3* using the housekeeping gene *eif5* as a control in single female carcass, together with positive (pool of adult male flies) and negative controls (pool of adult female flies) (electronic supplementary material, figure S5*b*). Neuroblasts from *X^X/Y* were combined into two biological replicates with five individuals in each, and submitted for RNA-seq.

(d) RNA-seq library preparations

Total RNA was isolated with TRIzol, mRNA purified twice with magnetic beads linked to poly-T tails (Dynabeads® mRNA DIRECT™ Purification Kit). The quality of mRNA preparations was accessed with the Agilent 2100 Bioanalyzer system (Agilent Technologies). Samples were subjected to library synthesis using the automated Apollo 324™ NGS Library Prep system with PrepX RNA-Seq kit (Wafergen). The final product of each library was submitted to 15 cycles of PCR enrichment in reactions containing one barcode for each sample. Final libraries were purified with magnetic beads to an average size of 350 bp, checked with Agilent 2200 TapeStation (Agilent Technologies), and submitted to 150 cycles of paired-end sequencing (2 × 150 bp) using HiSeq 2500 (Illumina). Two biological replicates were sequenced for each sample. Sequencing data are available at GEO with the accession number GSE93699.

(e) Mapping and analysis

Raw Illumina reads were trimmed using Trimgalore under default quality parameters (-q 30 –phred33). RNAseq trimmed reads were mapped against the reference genome (annotation 6.07) using bowtie2 under-sensitive option. Samtools was used to convert between SAM and BAM files and to sort BAM files. We used GFold function 'count' to extract read counts for annotated genes from sorted BAM files according to the GTF file (annotation 6.07). Read counts (electronic supplementary material, table S2) were used to calculate differential expression with DESeq2 (version 1.8.1), R software (version 3.2.3) and

RStudio (version 0.99.467) and subjected to quality control (electronic supplementary material, figures S6–S8). We conducted separate contrasts for the lines carrying the free Y chromosomes [*Y(c)* and *Y(o)*] and combined *p*-values across experiments with Fisher's method [40]. Finally, *q*-values were calculated with the *q*-value package (http://qvalue.princeton.edu/). Biological functions of genes differentially expressed were assessed through study of gene ontology using the online tool GOrilla (http://cbl-gorilla.cs.technion.ac.il/) [41,42].

(f) Digital PCR for mitochondrial DNA copy number

Adult flies (2-day-old) were used. Samples used for copy number analysis were treated with RNase A and normalized with Qubit 3.0 Fluorometer. DNA dilutions were made based on the estimated abundance of targets, and copy number assessed using BioRad's digital PCR.

(g) FlyAtlas

Microarray data were downloaded from FlyAtlas (http://flyatlas.org/data.html) and converted to log10 scale. In order to evaluate the tissue of origin of the differentially expressed genes (DEGs), the set of DEGs in the whole body of *X^X/Y* flies was merged with the FlyAtlas expression data. The expression average of upregulated genes was calculated for each tissue and illustrated with a Radar Plot; the values along the radial axis represent the average expression (arbitrary units of microarray signal) of the DEGs in each tissue. To identify tissue-specific genes, we calculated *Z* scores for expression values of each adult tissue for a total of 14 621 probes. Using a *Z* score cut-off greater than 3.5, we identified 4440 probes with expression in a single tissue. The association between DEGs and tissue-specific expression was assessed with Fisher's exact test. For the correlation plot, DEGs in the ovary of *X^X/Y* were identified and combined with the FlyAtlas. Pearson correlation between tissues was calculated with the 'cor' function and plotted with the library 'corrplot'. Fold changes obtained with DESeq2 were used to create density plots in 'ggplot2'. All data were processed using the R software (v. 3.2.3) and RStudio (v. 0.99.467).

3. Results and discussion

We constructed *Drosophila* strains that differ only in the presence/dose of the Y chromosome (electronic supplementary material, figures S1–S5) but that were otherwise genetically identical for all other chromosomes and mitochondria. The presence of the Y chromosome impacted female whole-body gene expression, whereas an extra Y chromosome had limited impact on males (figure 1). Accordingly, in the contrast between *X^X/0* versus *X^X/Y* flies we observed 54 up- and 77 downregulated genes ($q < 0.10$). Y chromosome presence in females preferentially affected genes expressed in the ovaries (figure 1*b*), with nine times more ovary-specific genes than expected by chance (electronic supplementary material, figure S9; $p < 0.001$, Fisher's exact test). We also detected mild enrichment for candidates expressed in midgut and salivary glands (electronic supplementary material, figure S10). As a control, we investigated male *X^Y/0* and *X^Y/Y* genotypes that are genetically identical except for the extra Y chromosome; the data showed a mostly stable mRNA profile in these flies (six up- and no downregulated genes; $q < 0.10$). Next, we dissected ovaries from *X^X/0* and *X^X/Y* flies as well as testes from *X^Y/0* and *X^Y/Y* males. We observed 383 DEGs between dissected ovaries of *X^X/0* and *X^X/Y* (92 up- and 291 downregulated genes; $q < 0.10$), whereas we found negligible differences between dissected testis of *X^Y/0* and *X^Y/Y* (18 up- and six downregulated genes; $q < 0.10$). The data suggested that female-exclusive tissues were acutely impacted by the presence of a Y chromosome. These observations are partially expected due to the limited opportunity for natural selection to act on Y chromosome genetic effects that impact female-exclusive tissues.

Direct Y chromosome effects in somatic tissues could also be functionally significant. The brain is a well-known organ with sexual dimorphism clearly documented from insects to mammals [29]. To address the consequence of a male-adapted chromosome in neuronal female cells, we studied genome-wide expression of mRNA in CNS of larvae carrying a Y chromosome. CNS from third instar larvae present a more homogeneous cell population than adult heads and were carefully dissected [43]; the procedure was efficiently performed, with the average time spent to dissect each larva at approximately 1 min (electronic supplementary material, figure S4). Matched individual carcasses were saved to identify male and female larvae (electronic supplementary material, figure S5). We detected a significant impact of Y chromosome presence in dissected neuronal tissues, with 108 up- and 213 downregulated genes in *X^X/Y* ($q < 0.10$; figure 1); notably, the set of downregulated genes was enriched for candidates involved in 'behaviour' (greater than threefold enrichment; $p < 0.001$ (*mHG*)) (figure 2 and electronic supplementary material, table S1), including 'mating behaviour' (greater than fivefold enrichment; $p < 0.001$ (*mHG*)) and 'reproductive behaviour' (greater than fivefold enrichment; $p < 0.001$ (*mHG*)) (electronic supplementary material, table S1). In addition, genes that control synaptic transmission were also significantly repressed by the Y chromosome. For instance, we detected repression of 69 out of 93 genes whose protein products are localized at the pre-synapsis (GO:0098793) ($p <$ 0.001 (Fisher's exact test)) and 38 out of 47 genes whose protein products are localized at the post-synapsis (GO:0098794) ($p <$ 0.001 (Fisher's exact test)) (figure 2). The Y chromosome also impacted other functional classes controlling neuronal signalling (figure 3; electronic supplementary material, figures S11 and S12). For instance, we observed the downregulation of genes involved in dopamine metabolism in *X^X/Y* flies (figure 3). This pathway comprises 12 genes, six of which were significantly inhibited by the presence of the Y chromosome (*Black*, *Ddc*, *Dop1R1*, *Dop1R2*, *Ebony*, and *Punch*). Dopamine signalling in flies, as well as in mammals, is associated with motor coordination, motivation, reward, addiction, learning and memory [44–51]. Among genes repressed in the CNS of *X^X/Y* genotypes, we also noted candidates involved in neuronal development. For instance, two out of four *Drosophila* genes encoding *Down syndrome cell adhesion molecules* (*Dscam*), a group of proteins implicated in the neurological phenotypes associated with Down's syndrome, were significantly repressed by the presence of the Y chromosome ($q < 0.10$). These genes are conserved in vertebrates and can be transcribed in tens of thousands of isoforms by alternative splicing in *Drosophila* [52–54]; this repertoire regulates dendritic elaboration or axonal arborization through homophilic repulsion [55,56]. The mechanism is the basis of self-avoidance that allows axonal and dendritic processes to uniformly cover their synaptic fields. Collectively, the data indicated that Y chromosome presence in female neuronal tissues exert

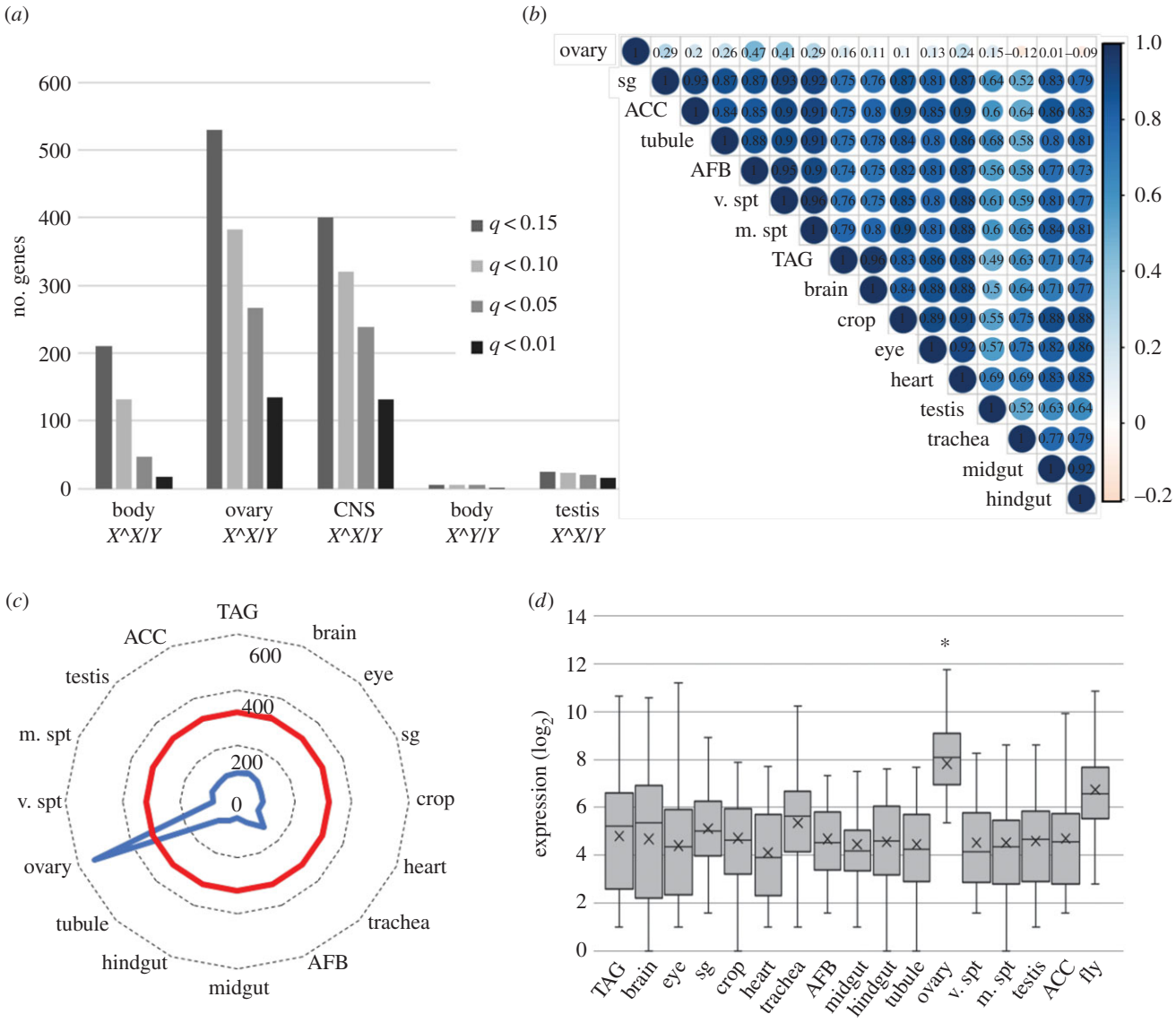

Figure 1. Y chromosome presence in females modulates ovary-specific genes. (*a*) Number of DEGs in each contrast and across four *q*-value thresholds (electronic supplementary material, tables S3–S7). (*b*) Plot shows the Pearson correlation between tissues for the set of genes induced in *X^X/Y* body (see methods); note that these genes have a unique expression pattern in ovary with very low correlation with the other tissues. (*c*) The set of genes induced in *X^X/Y* body are significantly enriched for highly expressed ovary-specific genes. The red line indicates the average expression of all genes in whole body preparations (FlyAtlas). The blue line represents the transcription average in each tissue. The scale of the radial axes is an arbitrary unit of microarray intensity (FlyAtlas). (*d*) Boxplot with expression level across tissues for the set of *X^X/Y*-induced genes in body. An asterisk (*) denotes $p < 0.05$ (Student's *t*-test), whiskers define maximum and minimum values, and 'x' denotes the mean of expression ($\log_2$ of arbitrary unit of microarray intensity) for each tissue. Plots (*b*–*c*) using genes identified with $q < 0.10$ in whole body *X^X/Y*; using other *q*-values yielded similar results. (Online version in colour.)

significant impacts on specific pathways, and raise the possibility that sex-specific adaptations linked to Y chromosome function might be partially responsible for the patterns.

The Y chromosome and the mitochondria are asymmetrically selected and expected to be hotspots for sex-specific adaptation [5,8,12,57–60]. A consequence of sex-specific evolution is sex-specific disruption when a genetic element that evolves in one sex exerts impacts on the other sex. For instance, mtDNA variation exerts a disproportionate impact on gene expression in male reproductive tissues [12]. Regulatory interactions between the Y chromosome and the mitochondria have been observed before [13] and might be gauged through changes in mitochondrial gene expression or copy number. We evaluated the transcription of all 33 genes encoded by the mitochondrial genome in female genotypes carrying the Y chromosome. The analysis revealed that the expression of mitochondrial genes in the reproductive tissue was strongly repressed (figure 4). All 33 genes were downregulated in the ovary of *X^X/Y* genotypes ($p < 0.001$, Fisher's exact test), with most genes repressed at greater than 1.5-fold change. The shift towards repression of mitochondrial genes was also significant in the CNS ($p < 0.05$, Fisher's exact test). Conceivably, lowered mtDNA copy number could explain reduced expression of mitochondrially encoded genes. However, analysis with quantitative digital PCR for genes in the mitochondrial genome (*Cox-1* and *16S*) showed that copy number of the mtDNA was undistinguishable between female strains with and without the Y chromosome (electronic supplementary material, figure S13). Collectively, the data supports the hypothesis that cryptic Y-linked adaptations exert their function through genetic interactions with the mitochondria, and indicates that Y-mitochondria interaction is mediated through transcriptional changes rather than reduction in the number of mtDNA copies.

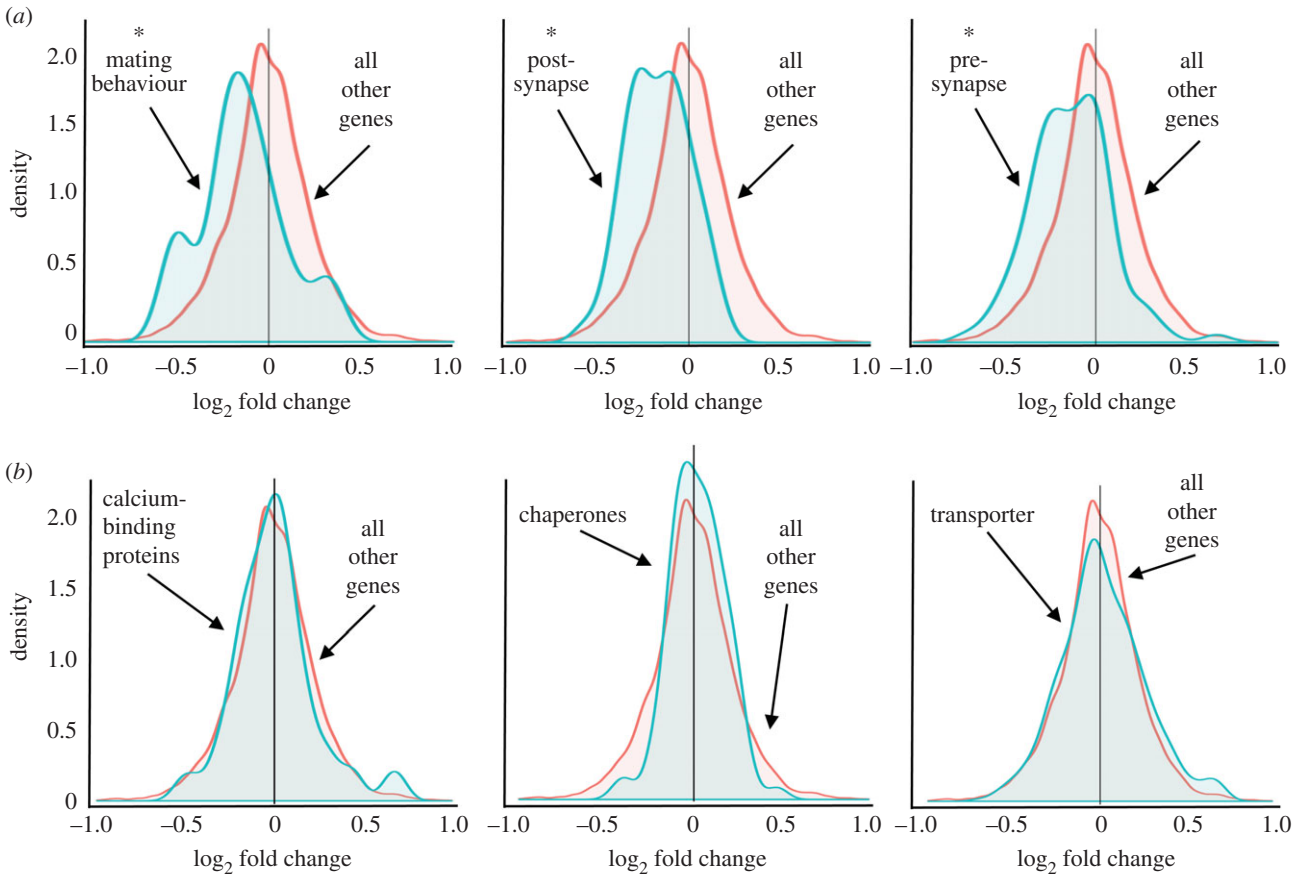

Figure 2. Y chromosome presence in female neuronal tissues induces the downregulation of genes involved in reproductive behaviour. (*a*) Density plot with fold change (contrast between *X^X/0* versus *X^X/Y*, RNA-seq in neural tissues) of genes involved in female mating behaviour (GO:0060180), or localized to the post-synapsis (GO:0098794) or pre-synapsis (GO:0098793). More than 96% of the genes are not shared between these classes (i.e. unique to each class). All classes are significantly repressed in *X^X/Y* relative to *X^X/0* genotypes (Kolmogorov–Smirnov, $*p < 0.05$ for all classes). (*b*) Equivalent density plot for the same comparison showing three other functional classes that are not affected by Y chromosome presence (Kolmogorov–Smirnov, $p > 0.05$ for all classes). (Online version in colour.)

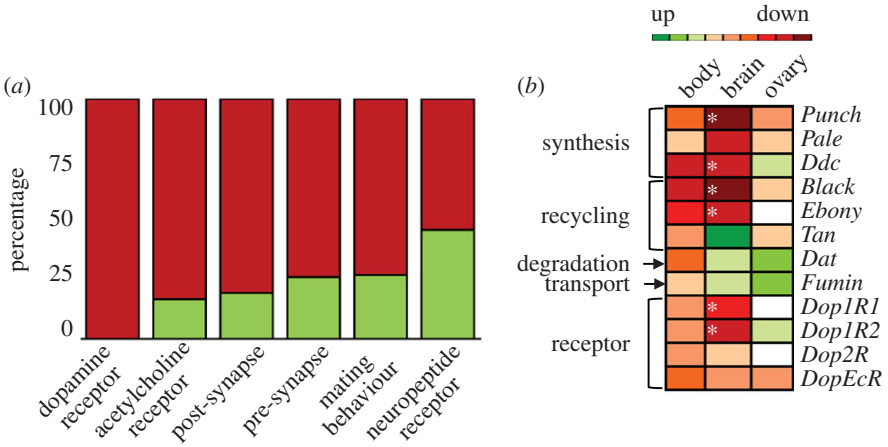

Figure 3. Downregulation of dopamine synthesis pathway in neural tissue with a Y chromosome. (*a*) Repression of gene transcription is observed across specific pathways of *X^X/Y* relative to *X^X/0* (data from RNA-seq of neural tissue). The plot displays the percentage of genes repressed (red) and induced (green) in each category. Except for neuropeptide receptors, all gene sets display significant bias towards downregulation in *X^X/Y* genotypes ($p < 0.05$, Fisher's exact test). (*b*) Heat map illustrates transcriptional changes in genes of the dopamine pathway. The contrast between *X^X/Y* relative to *X^X/0* genotype shows significant downregulation of six genes in CNS of *X^X/Y* strain (white stars denote $q < 0.10$). (Online version in colour.)

The Y chromosome has been implicated in natural variation of a number of phenotypes [13–15,61–63]. The phenotypic variation occurs despite lack of diversity in Y-linked protein-coding genes and it is presumably mediated by molecular mechanisms involving repetitive element variation and their influence on gene expression regulation [1,64–66]. One model predicts that repetitive Y-linked elements that require silencing by DNA-binding proteins generate a 'chromatin sink' with genome-wide impact on the balance between euchromatic (active) and heterochromatic (repressive) domains [13,67,68]. Thus, it could be expected that an extra dose or presence of the Y chromosome would modify the

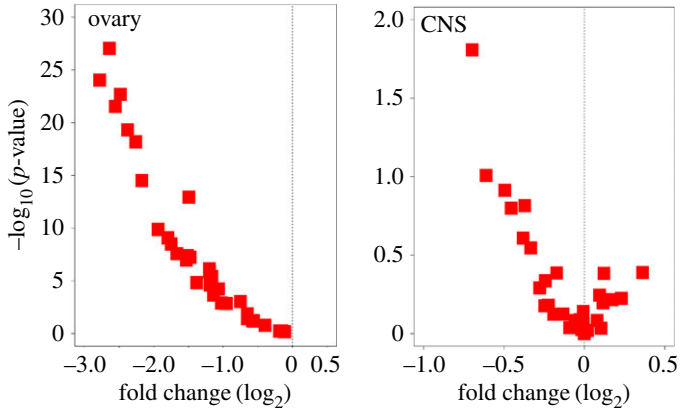

Figure 4. Y chromosome presence suppresses the expression of mitochondria-encoded genes in reproductive and somatic tissues. Volcano plot of all 33 genes encoded by the mitochondrial genome in ovary and CNS of *X^X/Y* flies reveal a concerted downregulation of mitochondrial genes. The volcano plots show fold change (*x*-axis) and $-\log_{10}$(*p*-value) (*y*-axis) for each gene (red dot); as expected, the shift towards downregulation of mitochondria-encoded genes was more salient in the reproductive tissue (ovary: $p < 0.001$ (Fisher's exact test)) than in the CNS (CNS: $p < 0.05$ (Fisher's exact test)). Y chromosome presence did not impact mtDNA copy number in *X^X/Y* relative to *X^X/0* (electronic supplementary material, figure S13). (Online version in colour.)

expression of genes normally silent [1]. We examined the expression of genes located in repressive chromatin domains [69], and found that their expression was unchanged in whole body (male and female), testis and ovaries of flies with Y chromosome aneuploidy; only DEGs in the CNS dataset were enriched for candidates located within repressive chromatin (electronic supplementary material, figure S14). We also evaluated the transcription of 12 functionally active genes buried within heterochromatin (*concertina*, *light*, *rolled*, *Dbp80*, *Nipped-B*, *Ribosomal protein L15*, *Ribosomal protein L38*, *Nipped-A*, *chitinase-3*, *l(2)41Ab*, *unextended*, and *Yeti*) and found that in all cases their expression was undistinguishable from the control (electronic supplementary material, figure S15). Collectively, our results indicate that the dose/presence of the Y chromosome had limited influence on genes localized within repressive chromatin domains. Instead, the data suggest functional responses to the Y chromosome.

The Y chromosome can exert important evolutionary roles in both reproductive and somatic tissues. Typically, the influence of the Y chromosome is most pronounced during male gametogenesis, such as the case when it triggers X-chromosome DNA breaks and lowers the viability of sperm carrying the X chromosome [6,70–72]. In mammals, the influence of the Y chromosome has also been suggested to occur in females through epigenetic modifications on the genetic material of sperm cells carrying the X chromosome [73]. Accordingly, polymorphic Y-linked effects were transmitted to the female progeny with impacts on the severity and onset of autoimmune phenotypes in the adult [73]. However, the consequences of Y chromosome presence in female tissues or the sensitivity of gene expression to the presence of an extra Y chromosome have remained unaddressed. This has been partially because of the lack of suitable genetic models to address direct genomic responses to Y chromosome presence, absence and number. Here, we show that *Drosophila* males were mostly insensitive to the presence of an extra Y chromosome whereas the presence of the Y chromosome in females exerted a significant impact in the ovary with substantial downregulation of mitochondrial genes. The presence of the Y chromosome in females also exerted functionally coherent impacts on the CNS. The observation that genes implicated in reproductive behaviour are significantly downregulated by Y chromosome presence is intriguing. The data indicated that both pre-synaptic and post-synaptic genes are affected even though less than 3% of the genes are shared between these two sets. Our observations provide a framework to address direct Y chromosome modulation of female genome function while controlling for genetic variation in all other genetic elements in both reproductive and somatic tissues.

Ethics. The authors declare that there are no ethical issues associated with this research.

Data accessibility. Data supporting this study have been uploaded as electronic supplementary material and are available through the GEO/SRA database.

Authors' contributions. A.T.B. and B.L. conceived and designed the study. A.T.B. and R.M.B. conducted the research. A.T.B., R.M.B. and B.L. analysed data. A.T.B. and B.L. wrote the manuscript.

Competing interests. We declare we have no competing interests.

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