



Tamoxifen induction of Cre recombinase does not cause long-lasting or sexually divergent responses in the CNS epigenome or transcriptome: implications for the design of aging studies

Ana J. Chucair-Elliott · Sarah R. Ocanas · David R. Stanford · Niran Hadad · Benjamin Wronowski · Laura Otalora · Michael B. Stout · Willard M. Freeman 

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Abstract The systemic delivery of tamoxifen (Tam) to activate inducible CreERT2-*loxP* transgenic mouse systems is now widely used in neuroscience studies. This critical technological advancement allows temporal control of DNA-cre recombination, avoidance of embryonically lethal phenotypes, and minimization of residual cell labeling encountered in constitutively active drivers. Despite its advantages, the use of Tam has the potential to cause long-lasting, uncharacterized side effects on the transcriptome and epigenome in the CNS, given its mixed estrogen receptor (ER) agonist/

antagonist actions. With the welcome focus on including both sexes in biomedical studies and efforts to understand sex differences, Tam administration could also cause sexually divergent responses that would confound studies. To examine these issues, epigenetic and transcriptomic profiles were compared in C57BL/6 J female and male hippocampus, cortex, and retina 1 month after a 5-day Tam treatment typical for cre induction, or vehicle control (sunflower seed oil). Cytosine methylation and hydroxymethylation levels, in both CG and non-CG contexts, were unchanged as

Ana J. Chucair-Elliott and Sarah R. Ocanas contributed equally to this work.

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A. J. Chucair-Elliott · S. R. Ocanas · D. R. Stanford · N. Hadad · B. Wronowski · L. Otalora · M. B. Stout · W. M. Freeman

Reynolds Oklahoma Center on Aging, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

A. J. Chucair-Elliott · S. R. Ocanas · D. R. Stanford · L. Otalora · W. M. Freeman (✉)

Department of Physiology, University of Oklahoma Health Sciences Center, 975 NE 10th Street, Oklahoma City, OK 73104, USA

e-mail: wfreeman@ouhsc.edu

N. Hadad · W. M. Freeman

Oklahoma Nathan Shock Center for Aging, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

N. Hadad · W. M. Freeman

Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

M. B. Stout

Department of Nutritional Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

W. M. Freeman

Oklahoma City Veterans Affairs Medical Center, Oklahoma City, OK, USA

determined by oxidative bisulfite sequencing. Long-lasting Tam transcriptomic effects were also not evident/minimal. Furthermore, there is no evidence of sexually divergent responses with Tam administration and Tam did not alter sex differences evident in controls. Combined with recently reported data that Tam alone does not cause long-lasting changes in behavior and neurogenesis, our findings provide confidence that Tam can be used as a cre-recombinase inducer without introducing significant confounds in transcriptomic and epigenomic neuroscience studies, particularly those focused on genomic and transcriptomic aspects of the aging brain.

Keywords Transgenic · Tamoxifen · Sex differences · Epigenome · Methylation · Transcriptome · Hippocampus · Cortex · Retina

Introduction

Epigenetic mechanisms, such as chromatin structure and DNA modifications, affect DNA accessibility and act as regulators of genomic organization and gene expression. Methylcytosine (mC) and hydroxymethylcytosine (hmC) can be dynamic or long-lasting DNA modifications that can also be passed on to daughter cells (Holliday 2006; Law and Jacobsen 2010; Allis and Jenuwein 2016; Masser et al. 2018). Recently, mC and hmC modifications have become a focus of neuroscience and aging research studies, along with other fields. Of particular interest in aging studies, specific genomic sites of differential methylation patterns in human and animal models have been identified (Horvath et al. 2012; Lister et al. 2013; Rakyan et al. 2010; Maegawa et al. 2010; Masser et al. 2017). Also, as biomedical research has begun to address the underrepresentation of female animal models in studies [(Clayton and Collins 2014)], developmental sex divergences in CNS DNA methylation have been identified (Nugent et al. 2015). Moreover, sex differences in hippocampal CG and non-CG (CH) methylation are evident in early adulthood and persist into advanced age in mouse and man (Masser et al. 2017). In addition to differences in the epigenome, sex-specific transcriptome profiles are evident. For instance, sexually divergent expression of the MHCII pathway across the CNS with aging (Mangold et al. 2017a), and significant sex differences in gene expression in the retina (Du et al. 2017)

throughout the life span have been reported. Two understudied neuroscience questions are (1) whether sex-dependent differences in the epigenome and transcriptome are cell type-specific and (2) how sex affects DNA modifications and transcriptomic profiles with brain aging. The development of cell type-specific reporter *Cre-lox* mouse models that label specific cell types and allow paired analysis of the methylome and transcriptome upon induction of cre-recombinase with Tam could make the study of these questions feasible. Yet, if and how the introduction of Tam as an inducer of recombination affects the epigenetic and transcriptional processes in the brain has not been addressed. These control studies are needed for the field to have confidence that Tam induction models are not introducing a confounding variable.

Tam is a selective estrogen receptor modulator (SERM) that exerts agonist or antagonist activity on estrogen receptors (ERs) in a tissue-specific manner. This response depends on the complexity of ER signaling, its distribution, ligand-binding specificity, and interactions with co-activators or co-repressors (Pfaffl et al. 2001; Tee et al. 2004; Kuiper et al. 1997; Bramlett and Burris 2002; Webb et al. 2003). Since the early 1970s, Tam has been widely used as a peripheral ER antagonist for the treatment of breast cancer while identified as an ER agonist associated with increased endometrial cancer [(Maximov et al. 2013; van Leeuwen et al. 1994; Fornander et al. 1989)]. Within the brain, Tam may have ER-mixed antagonist-agonist properties (Newhouse and Dumas 2015; Haynes and Dowsett 1999), possibly due to expression of classical ERs by glial cells (Garcia-Ovejero et al. 2005), enhanced under several pathological conditions (Khalaj et al. 2013; Sakuma et al. 2009; Takahashi et al. 2004; Lu et al. 2003; Blurton-Jones and Tuszyński 2001). The expression of ER α has been localized in the cellular processes and soma of microglial cells in the hippocampus of adult mice and rat cerebellum, consistent with non-classical and understudied mechanisms of action of ERs (Marin et al. 2006; Beyer et al. 2003; Suuronen et al. 2005).

During the last two decades, Tam has become an important reagent applied in the analysis of gene functions, as well as cell-specific tagging in inducible conditional mouse mutants (Indra et al. 1999; Srinivasan et al. 2016; Jahn et al. 2018; Fonseca et al. 2017; Feil et al. 1997). Temporally controlled recombination of

floxed target genes achieved by the CreERT2/loxP system circumvents embryonic lethal or aberrant phenotypes associated with constitutively activated cre recombinase during development (Jahn et al. 2018; Patel et al. 2017). CreERT2 is a recombinant fusion protein of the cre DNA recombinase and a mutant ligand-binding domain of the human ER. Similarly, to the endogenous ER, CreERT2 is trapped in the cytosol by heat shock proteins. While this protein complex is impervious to natural ligands, it is highly sensitive to synthetic estrogen antagonists, such as the Tam active metabolite, 4-OH-Tam, which in turn activates the CreERT2 for recombination of floxed alleles in the cell nucleus. The cross of mice expressing cell-specific TAM-inducible cre recombinases with floxed allele mice such as INTACT (isolation of nuclei tagged in specific cell types) [(Mo et al. 2015)], TRAP (translating ribosome affinity purification) (Heiman et al. 2008), RiboTag (Sanz et al. 2009), and most recently developed NuTRAP (nuclear tagging and translating ribosome affinity purification) (Roh et al. 2017), are promising approaches for interrogating the sex- and age-dependent differences in transcriptomes and epigenomes of specific cell types in the adult CNS. In these models, upon Tam treatment, the tagging of cell-specific nuclei and/or ribosomes and downstream isolation of nucleic acids is only possible in floxed mice that co-express a specific cre transgene but not in the cre-negative counter-parts. In this context, the execution of parallel cre-negative controls is no longer a viable control as there will be no tagged ribosomes or nuclei for isolation.

A potential disadvantage of the use of Tam as inducer of CreERT2 in neuroscience research is the possibility of long-term effects on the normal brain function, proteins, or nucleic acids. In regard to this concern, Tam metabolism in mouse brains was reported to be age-, strain-, and dose-dependent (Patel et al. 2017). Another study suggested neither the route of application nor sex of treated mice influenced the response to Tam, and based on an end-point analysis 15 days post-treatment, concluded that short Tam treatments used to activate CreERT2 models have no significant persistent impact on adult neurogenesis or behavioral testing (Rotheneichner et al. 2017).

The purpose of the present study is to examine the long-lasting effects of Tam on the existing sex differences in the transcriptome as well as in genomic methylation and hydroxymethylation patterns in the brain. To

do so, and in an attempt to separate the effect of Tam from the effects of Cre- or floxed-transgene expression, we performed paired analysis of cytosine methylation and hydroxymethylation levels, in both CG and non-CG contexts and RNA-seq transcriptomic profiles in hippocampus, cortex, and retina tissues from female and male C57BL/6 J mice treated with Tam or with its vehicle (Veh, sunflower seed oil).

No significant, long-lasting effects of Tam treatment in the transcriptome or epigenome were found in any of the tissues analyzed. Combined with recently reported data that Tam alone does not cause long-lasting changes in behavior and neurogenesis, our findings provide confidence that Tam can be used as a cre-recombinase inducer without introducing significant confounds in cell-specific transcriptomic and epigenomic neuroscience studies, including those exploring mechanisms to be targeted in aging (Unnikrishnan et al. 2017; Ashpole et al. 2017; Deepa et al. 2017; Podlutzky et al. 2017), where timely controlled tamoxifen induction could be of benefit in elucidating cell-specific contributions to the development or delay of any of the hallmarks of aging.

Methods

Animals

Male ($n = 8$) and female ($n = 7$) C57BL/6 J mice were purchased from the Jackson Laboratory (stock number: 000664; Bar Harbor, ME), housed in the animal facility at the University of Oklahoma Health Sciences Center and maintained under specific pathogen-free (SPF) conditions in a HEPA barrier environment. At 3 months of age, mice were divided into 4 experimental groups: female + Veh ($n = 3$), female + Tam ($n = 4$), male + Veh ($n = 4$), and male + Tam ($n = 4$) and subjected to Tam or Veh treatment. One month following Tam treatment, mice were euthanized, and hippocampi, cortices, and retina were harvested for further analyses. All procedures with mice were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Tamoxifen treatment

At 3 months of age, mice received a single daily intraperitoneal (ip) injection of 100 μ l Tam solubilized in 100% sunflower seed oil by sonication (100 mg/kg

body weight, 20 mg/ml stock solution, Sigma, St. Louis, MO) for five consecutive days (Srinivasan et al. 2016). Control mice received 100 μ l of Veh, 100% sunflower seed oil, by ip delivery.

Genomic DNA and total RNA isolations

Genomic DNA (gDNA) and total RNA (RNA) were isolated from hippocampal, cortical, and retinal frozen tissue using AllPrep DNA/RNA Mini kits (Qiagen, Germantown, MD) as described previously (Imperio et al. 2016; Mangold et al. 2017b). gDNA was quantified by Qubit fluorescent assays (dsDNA BR assay kit, ThermoFisher Scientific, Grand Island, NY) and RNA was quantified with a Nanodrop 2000c spectrophotometer (ThermoFisher Scientific). gDNA and RNA quality (> 8 DNA and RNA integrity numbers) were assessed by genomic DNA D1000 and RNA ScreenTape assays, with a 2200 TapeStation analyzer (Agilent Technologies, Santa Clara, CA).

Library construction and oxidative bisulfite sequencing

For each hippocampal, cortical, and retinal sample, 1 μ g of gDNA was brought up to 50 μ l volume with 1 \times low-EDTA TE buffer and sheared with a Covaris E220 sonicator (Covaris, Inc., Woburn, MA) to an average base pair size of 200 using the following settings: intensity of 5, duty cycle of 10%, 200 cycles per burst, 2 cycles of 60 s, at 7 $^{\circ}$ C. The size of sheared products was confirmed by capillary electrophoresis (DNA D1000, Agilent). gDNA fragments were cleaned by an Agencourt bead-based purification protocol, after which gDNA was quantified (Qubit, ThermoFisher Scientific). Two aliquots of 200 ng gDNA fragments were prepared in a 12 μ l volume to which 1 μ l of spike-in control DNA (0.08 ng/ μ l) with known levels of specific mC, hmC, and fC at individual sites was added. End repair, ligation of methylated adaptors (#L2V11DR-BC 1–96 adaptor plate, NuGEN, Tecan Genomics, Inc., Redwood City, CA) and final repair were performed according to manufacturer's instructions (Ovation Ultralow Methyl-Seq Library System (NuGEN). Of the two DNA aliquots per sample, one was oxidized and then bisulfite-converted and the other only bisulfite-converted with the True Methy oxBS module (NuGEN) with desulfonation and purification. Twenty-two microliters of libraries was eluted from the magnetic beads. qPCR was used to determine the number (N) of PCR cycles required for

library amplification. Bisulfite-converted samples were amplified for 7 cycles while oxidative bisulfite-converted samples were amplified for 11 cycles [95 $^{\circ}$ C—2 min, N (95 $^{\circ}$ C—15 s, 60 $^{\circ}$ C—1 min, 72 $^{\circ}$ C—30 s)]. Amplified libraries were purified with Agencourt beads and eluted in low-EDTA TE buffer. TapeStation HD1000 was used to validate and quantify libraries. Amplified libraries were normalized to a concentration of 4 nM and pooled, denatured, and diluted to 12 pM for sequencing on MiSeq (Illumina, San Diego, CA) according to manufacturer's guidelines with the exception of a custom sequencing primer (MetSeq Primer) that was spiked in with the Illumina Read 1 primer to a final concentration of 0.5 μ M.

OxBS-seq data analysis

Prior to alignment paired-end reads were adaptor-trimmed and filtered using FASTQ Toolkit in Basespace (Illumina, San Diego, CA). End-trimming removed 3 bp and 4 bp from the 5' and 3' end of each paired-end read, respectively. Only reads with a *Q* score \geq 25 and matching length criteria were used for mapping. Alignment of trimmed bisulfite-converted sequences was carried out using MethylSeq in Basespace (Illumina) (Krueger and Andrews 2011) against the mouse reference genome (GRCm38/mm10). Methylation call percentages for each CpG and non-CpG (CH) site within the genome were calculated by dividing the methylated counts over the total counts for that site in the oxidative bisulfite-converted libraries (OXBS). Genome-wide CpG and CH methylation levels were calculated separately. Hydroxymethylation levels in CpG (hmCG) and CH (hmCH) contexts were calculated by subtracting call levels from the oxidative bisulfite-converted (OXBS) libraries from the bisulfite-converted (BS) libraries. BAM files generated by MethylSeq (Basespace, Illumina) were run through MethylKit in R (Akalin et al. 2012) to generate context-specific (CpG/CH) coverage text files. Methylation percentages for the X and Y chromosomes were calculated separately as previously described [(Hadad et al. 2016)]. Briefly, coverage files were filtered to X and Y chromosomes to calculate sex-chromosome-specific methylation and hydroxymethylation levels in the same manner as genome-wide levels. Bisulfite conversion efficiency for C, mC, and hmC was estimated using CEGX spike-in control sequences (Supplemental Fig. 1). Untrimmed fastq files were run through CEGX QC v0.2

which output a `fastqc_data.txt` file containing the conversion mean for C, mC, and hmC.

Library construction and RNA-seq

Stranded RNA sequencing (RNA-Seq) (TruSeq v2, Illumina) was performed on 500 ng of total RNA from each sample and brain region from males and females, VEH- and TAM-treated mice ($n = 3\text{--}4/\text{group}$). Library construction was performed in a stranded manner to retain the directionality of the transcripts (Sultan et al. 2012). Each sequencing library was prepared from RNA from a single animal to provide biological variance. Illumina Truseq Stranded HT library generation was performed according to manufacturer's instructions. Briefly, polyA containing mRNA was purified using oligo-dT attached magnetic beads. mRNA was then chemically fragmented and cDNA synthesized. For strand-specificity, the incorporation of dUTP instead of dTTP in the second strand cDNA synthesis does not allow amplification past this dUTP with the polymerase. Following cDNA synthesis, each product underwent end repair process, the addition of a single 'A' base, and finally ligation of adapters. The cDNA products were further purified and enriched using PCR to make the final library for sequencing. Library sizing was performed by TapeStation (Agilent Technologies) and libraries were quantified by qPCR (Kappa Biosystems, Inc., Wilmington, MA). The cDNA library was then sequenced using an Illumina HiSeq 2500 at the PennState College of Medicine Genome Sciences Facility in a 2×100 bp fashion for the hippocampus. RNA-seq libraries from cortex and retina tissues were sequenced using an Illumina NextSeq (2×75 bp) at the Oklahoma Medical Research Foundation Genomics Facility.

RNA-Seq data analysis

Following sequencing, reads were trimmed, aligned, differential expression statistics and correlation analyses were performed in Strand NGS software package (Agilent). Reads were aligned against the Mm10 build of the mouse genome (2014.11.26). Alignment and filtering criteria included: adapter trimming, fixed 2 bp trim from 5' and 6 bp from 3' ends, a maximum number of one novel splice allowed per read, a minimum of 90% identity with the reference sequence, a maximum of 5% gap, trimming of 3' end with $Q < 30$. The alignment was

performed directionally with read 1 aligned in reverse and read 2 in forward orientation. Reads were filtered based on the mapping status and only those reads that aligned normally (in the appropriate direction) were retained. Normalization was performed with the DESeq algorithm [(Anders and Huber 2010)]. Transcripts with an average read count value > 20 in at least 100% of the samples in at least one group were considered expressed at a level sufficient for quantitation per tissue and those transcripts below this level were considered not detected/not expressed and excluded, as these low levels of reads are close to background and are highly variable. For statistical analysis of differential expression, a two-way ANOVA with the factors of sex and treatment and a Benjamini-Hochberg multiple testing correction followed by Student-Newman Keuls post hoc test was used. For those transcripts meeting this statistical criterion, a fold change $> |1.25|$ cutoff was used to eliminate those genes which were statistically significant but unlikely to be biologically significant and orthogonally confirmable due to their very small magnitude of change. Visualizations of hierarchical clustering and principal components analysis were performed in Strand Next Generation Analysis Software (NGS) (Version 3.1, Bangalore, India). The entirety of the sequencing data is available for download in FASTQ format from NCBI Sequence Read Archive (GSE135752 and GSE135208).

Quantitative PCR

Confirmation of gene expression levels was performed with quantitative PCR (qPCR) as previously described [(Du et al. 2017; Masser et al. 2014)]. cDNA was synthesized from purified RNA with the ABI High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) from the following RNA amounts: 1 μg from hippocampus, 500 ng from retina, and 200 ng from cortex. qPCR was performed with gene-specific primer-probe fluorogenic exonuclease assays (TaqMan, Life Technologies, Waltham, MA, Supplemental Table 1) and the QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems). Relative gene expression (RQ) was calculated with Expression Suite v 1.0.3 software using the $2^{-\Delta\Delta\text{Ct}}$ analysis method with GAPDH as an endogenous control. Statistical analysis of the qPCR data was performed using SigmaPlot 12.5 (SyStat Software, San Jose, CA). Two-way ANOVA analyses were performed with the factors of sex and treatment. Post-hoc All Pairwise

comparisons with factors of sex and treatment and interactions of both factors were performed with all pairwise multiple comparison procedures (Holm-Sidak method) with overall significance level = 0.05.

Protein extraction and western blotting

Hippocampus, cortex, and retina samples were harvested and homogenized in RIPA buffer (Thermo Scientific) supplemented with 1× Halt™ protease inhibitor cocktail (Thermo Scientific) by sonication (Sonic Dismembrator Model 100, Fisher Scientific). The supernatants from tissue homogenates were assayed for protein content using a BCA protein method (Thermo Scientific). Equal amounts of protein per sample (20 µg) were resolved on SDS 4–20% gradient polyacrylamide gels (Thermo Scientific) prior to transferring onto nitrocellulose membranes. Proteins were detected using the following primary antibodies: anti-BANP antibody (Abcam, Cambridge, MA, # ab105404, 1:2000), anti-FOS (Sigma-Aldrich, # SAB4500995, 1:1000), and anti-β actin (Abcam, #ab6276, 1:10,000). For secondary chemiluminescent detection, blots were incubated with corresponding HRP-conjugated secondary antibodies (GE Healthcare Bio-Sciences, Pittsburgh, PA). Imaging of the blots and analysis of band intensities were performed by conventional image analysis using a Kodak in vivo imaging system F Pro and Carestream MI SE 4.4 SE version software (Carestream Health, Inc., Rochester, NY) [(Chucair-Elliott et al. 2017)]. Two-way ANOVA analyses were performed with the factors of sex and treatment. Post-hoc all pairwise comparisons with factors of sex and treatment and interactions of both factors were performed with all pairwise multiple comparison procedures (Holm-Sidak method) with overall significance level = 0.05.

Results

For the study of the potential interactions of Tam systemic treatment with sex in the mouse brain at the epigenetic and transcriptomic levels, the terminology follows recommendations stated by McCarthy et al. (McCarthy et al. 2012). We define a sex difference as a difference between males and females that persists throughout life (such as gene expression level of a particular gene), a sexual dimorphism as a binomial difference between males and females (such as Y

chromosome encoded gene expression is only in males). Sex divergences are emergent, such as expression of a gene at a young age that is the same in males and females but becomes different at old age [(McCarthy et al. 2012)].

Previously observed X-linked sex differences and Y-linked dimorphisms are unaffected by tamoxifen

To test whether Tam had long-lasting effects on the existing sex differences/dimorphisms in brain gene expression, expression of genes located on the X- and Y-chromosomes [(Du et al. 2017; Armoskus et al. 2014; Snell and Turner 2018) was measured by qPCR in hippocampus, cortex, and retina samples from female and male C57BL/6 J mice 30 days after a 5-day course of Tam or Veh treatment.

The X-linked target genes selected were *Ddx3x*, with diverse roles in RNA splicing and export, translation initiation, cell cycle regulation, and apoptosis (Cordin et al. 2006; Su et al. 2015; Sun et al. 2008); *Kdm6a* (*Utx*), a histone lysine demethylase that catalyzes removal of H3K27me2 and me3, and possibly also functions as a transcriptional activator (Agger et al. 2007; Lee et al. 2007; Xu et al. 2008, 2006); *Eif2s3x*, which regulates the rate of protein translation (Xu et al. 2006); and *Xist*, necessary and sufficient to initiate X-inactivation (Gayen et al. 2016). As shown in Fig. 1, 1 month after cessation of treatment, the sex differences and divergences of all X-linked transcripts tested between males and females across hippocampus, cortex, and retina tissues was unchanged. Differential expression by sex was independent of treatment. Males treated with Veh had significantly lower expression of *Ddx3x*, *Kdm6a*, *Eif2s3x*, and no detectable expression of *Xist* compared to Veh-treated females. Tam treatment did not significantly affect such differences.

For the analysis of Y-linked genes, the following target genes were selected: *Ddx3y*, a Y chromosome homolog of *Ddx3x*, and regulator of spermatogenesis and sperm development in mice (Mazeyrat et al. 1998); *Kdm5d*, required for demethylation of H3K4me3 and H3K4me1 [(Blair et al. 2011; Mizukami et al. 2019)]; *Eif2s3y*, a Y chromosomal homolog of the X-linked *Eif2s3x* [(Abdelhaleem 2005)]; with role in spermatogenesis (Kopsida et al. 2009; Mazeyrat et al. 2001); and *Uty*, a *Utx* paralogue on the Y chromosome [(Xu et al. 2008)]. As previously shown in the mouse CNS (Du et al. 2017; Armoskus et al. 2014; Xu et al. 2008), such

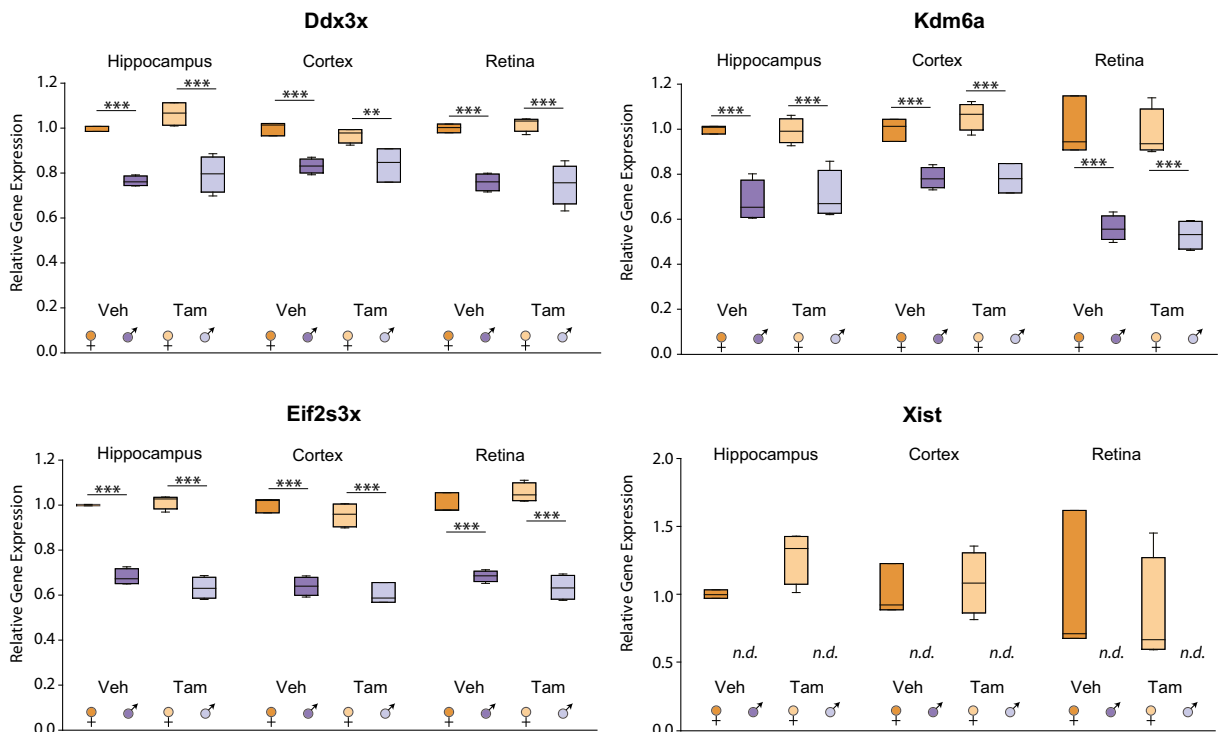


Fig. 1 Previously observed X-linked sex differences are unaffected by Tam. At 3 months of age, male and female C57BL/6 J mice ($n = 3-4$ /group) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. One month after cessation of treatment, hippocampus, cortex, and retina were harvested and RNA processed for qPCR analysis of X chromosome-linked

candidate genes. TaqMan probes were used to target *Ddx3x*, *Kdm6a*, *Eif2s3x*, *Xist* genes, and GAPDH as housekeeping gene. Box plot expresses average normalized RQ for each group \pm SEM. $**p < 0.01$, $***p < 0.001$, by two-way ANOVA with factors of sex and treatment followed by all pairwise multiple comparison procedures (Holm-Sidak method)

Y-linked genes are exclusively expressed in males. The expression of *Kdm5d* was slightly ($< 20\%$) and significantly lower in cortex and retina of Tam-treated male mice compared to controls. Similarly, Tam treatment resulted in slightly decreased *Uty* expression in male retina and hippocampus. Despite small changes in the transcription of two of the Y-linked genes with treatment, Tam did not generally affect the sexually dimorphic gene expression tested in the CNS (Fig. 2).

Tamoxifen does not interact with sex differences in the hippocampus, cortex, and retina

To examine the long-term effects of Tam treatment on the female and male transcriptomes across different regions of the CNS, a discovery approach was taken. Hippocampi, cortices, and retinae collected 30 days after cessation of ip treatment with Tam or Veh and were processed for RNA-Seq analysis.

An average of 19 million reads per sample passed filtering and alignment criteria for cortex and retina samples. Hippocampal samples were sequenced to a great depth averaging 44 million reads per sample. There was no difference in the number of reads mapped per group within each tissue and equivalent number of reads mapped to the positive and negative strands. Using a read count cutoff of > 20 for all samples in at least one group, 13,163 transcripts with mouse RefSeq annotations were called as expressed in the retina, 13,237 cortex, and 14,046 hippocampus. Principal component analysis (PCA) on samples for each tissue revealed no clustering by treatment and some segregation by sex, as expected (Fig. 3a–c). Differentially expressed transcripts between groups were identified through a two-way ANOVA, with factors of treatment and sex, followed by the Benjamini-Hochberg multicomparison test ($p < 0.05$). These transcripts were further filtered to only those that had a $> |1.25|$ fold difference between the groups that had a significant post-hoc test result. For

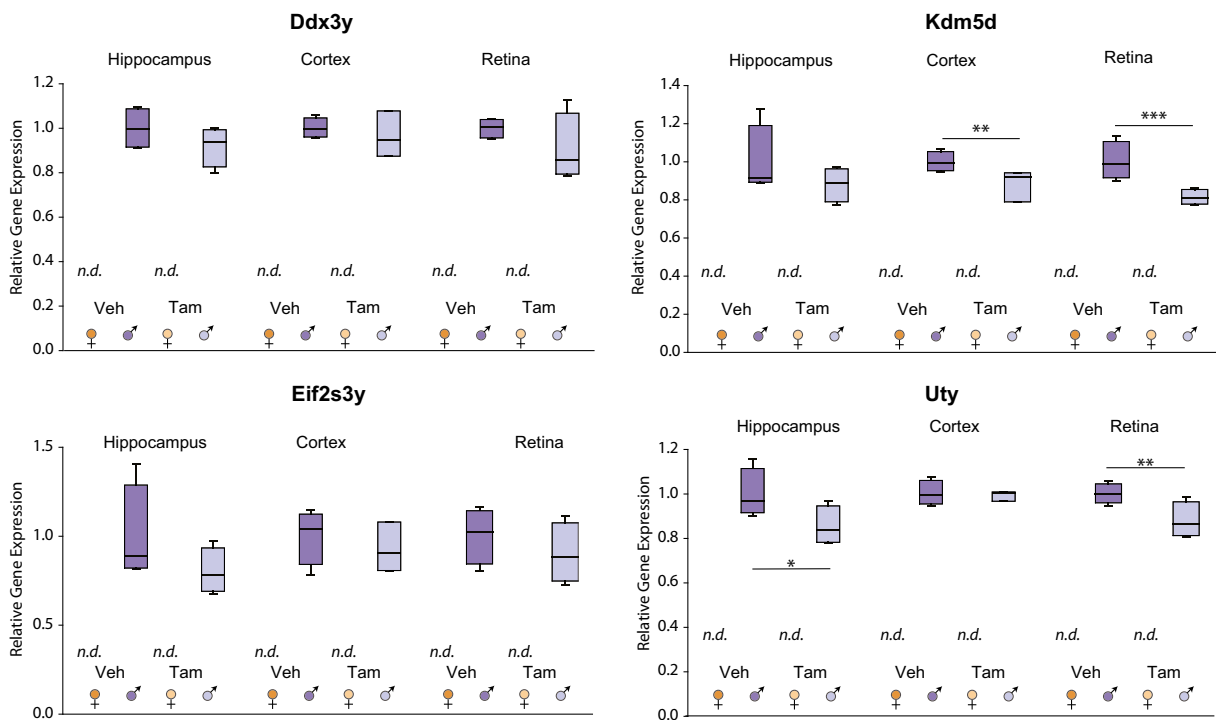


Fig. 2 Previously observed Y-linked sex dimorphisms are unaffected by Tam. At 3 months of age, male and female C57BL/6 J mice ($n = 3\text{--}4/\text{group}$) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. One month after cessation of treatment, hippocampus, cortex, and retina were harvested and RNA processed for qPCR analysis of Y chromosome-linked candidate genes. TaqMan assay probes were used to target *Ddx3y*,

Kdm5d, *Eif2s3y*, subunit 3, and *Uty* transcripts. Box plots express average normalized RQ for each group \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparing male and female groups under the same treatment by two-way ANOVA followed by all pairwise multiple comparison procedures (Holm-Sidak method). *n.d.* expression not detected

hippocampus, 2073 genes by sex and no significant differences with treatment or for an interaction effect were found. Similarly, hierarchical clustering on the hippocampus samples demonstrated that the individual samples were separated by sex with samples from Tam- and Veh-treated mice interspersed by sex (Fig. 3d).

As previously published for cortex (Mangold et al. 2017b) and retina (Du et al. 2017), the sex differences in the transcriptome were maintained across tissues and treatments in our study. There were no significant interactions between Tam treatment and sex in either of these CNS tissues (Fig. 4). Cortex differentially expressed 8 genes for sex (Fig. 4a, c) and 11 genes for treatment (Fig. 4e). The number of genes differentially expressed in the retina was 10 for sex (Fig. 4b, d) and 2 for treatment (Fig. 4f). Collectively, the RNA-seq analysis identified no significant interaction effect of Tam treatment with sex differences and only minimal effects of Tam administration.

Tamoxifen has minimal effect on the expression of selected autosomal transcripts across hippocampus, cortex, and retina tissues

To further assess potential interactions between Tam and sex shown by our RNA-seq results, differentially expressed autosomal genes with Tam administration in the cortex and retina were examined by qPCR. For cortex, the target genes were *Egr2*, associated with the onset of myelination of the peripheral nervous system and hindbrain development and for T and B cell development and differentiation [(Taefehshokr et al. 2017)]; *Fos*, which helps the formation of the transcription factor AP-1 to regulate a wide array of genes in response to many stimuli [(Coulon et al. 2010)]; and *Arc*, implicated in neuronal activation, synaptic plasticity, and a variety of memory tasks (Bramham et al. 2008; Gao et al. 2018) (Fig. 5a). For retina, the target genes were *Banp*, which functions as anti-tumorigenic and

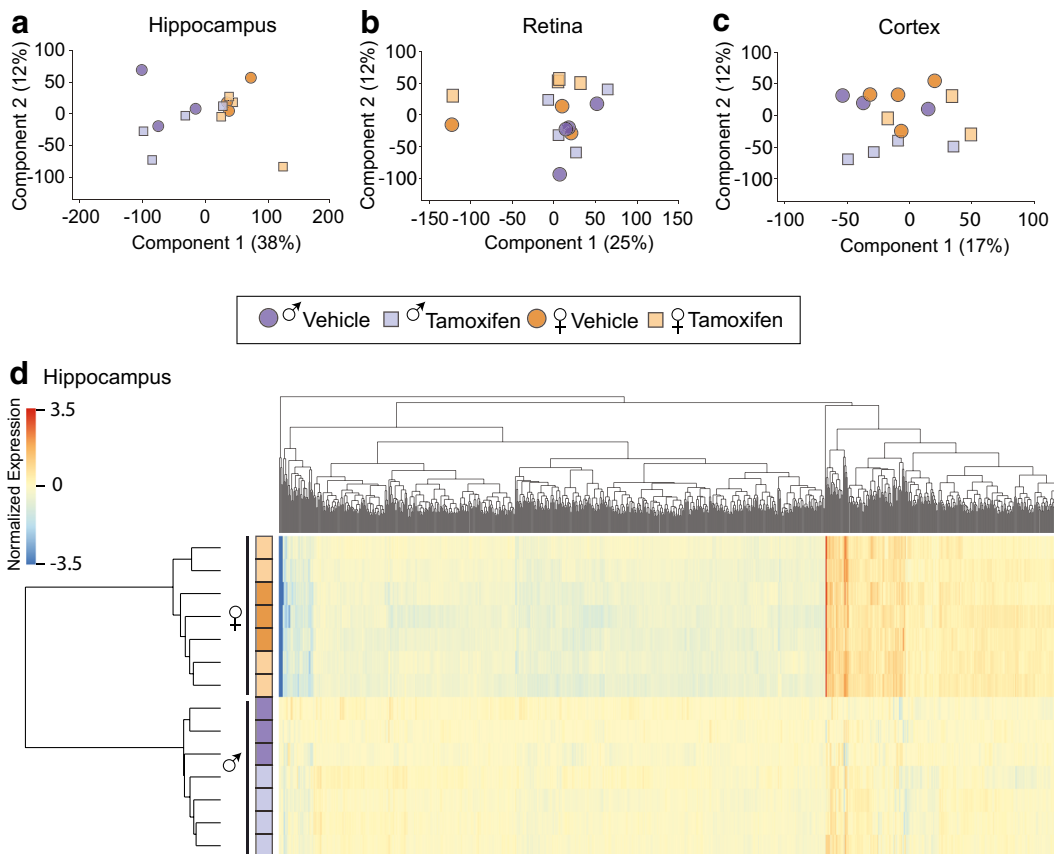


Fig. 3 Tam does not interact with sex differences in hippocampus, retina, or cortex. At 3 months of age, male and female C57BL/6 J mice ($n = 3\text{--}4/\text{group}$) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. Following 1 month after cessation of treatment, their CNS tissues were harvested and processed for RNA-Seq analysis. **a** Principal component analysis (PCA) of transcriptome profile of hippocampus shows the consistent sex differences we have described previously but Veh- and Tam-treated samples cluster together. PCA of retina (**b**) and cortex

(**c**) did not show clustering by treatment, with female and male samples interspersed with treatment. **d** Heatmap presentation of genes with significant differences in expression analysis (two-way ANOVA, factors of treatment and sex, $p < 0.05$, Benjamini-Hochberg multiple testing correction) revealed 2073 genes differentially expressed by sex and no significant differences with treatment or for an interaction effect in the hippocampus. By hierarchical clustering samples were separated by sex with samples from Tam- and Veh-treated mice interspersed by sex

modulator of the cell cycle through interaction with and stabilization of p53 [(Malonia et al. 2011)]; and *Map3k13*, reported to activate both the NF κ B and JNK pathways (Keshet and Seger 2010) (Fig. 5b). The qPCR data analysis showed significantly higher expression of *Egr2*, *Arc*, and *Fos* genes in the cortex of Tam-treated males and/or females compared to their same-sex Veh-treated counterparts, with only sex and treatment interactions for *Fos* expression (Fig. 5a). In the retina, Tam had a slight effect decreasing the transcript levels of *Banp* compared to same-sex control groups. *Map3k13* transcription was slightly lower in Tam-treated females compared to their respective VEH-control (Fig. 5b). For hippocampus, no autosomal genes were found to be

differentially expressed with treatment by RNA-seq, thus no qPCR data is shown.

In order to assess whether the minimal effect of Tam on the gene expression of selected autosomal transcripts has repercussions at the protein level, WB was performed on cortex and retina protein extracts from all experimental groups. For the protein analysis, and based on significance of changes by qPCR, *Fos* was selected for cortex, and *Banp* for retina. In cortex, a single *Fos* detected protein band showed at ~ 55 kDa, consistent with predicted size (Fig. 5c). Of note, for *Banp* (with an expected band size of ~ 56 kDa on WB of specific cell lysates and tissues, and with variable reported sizes from 50 to 71 KDa), in retina

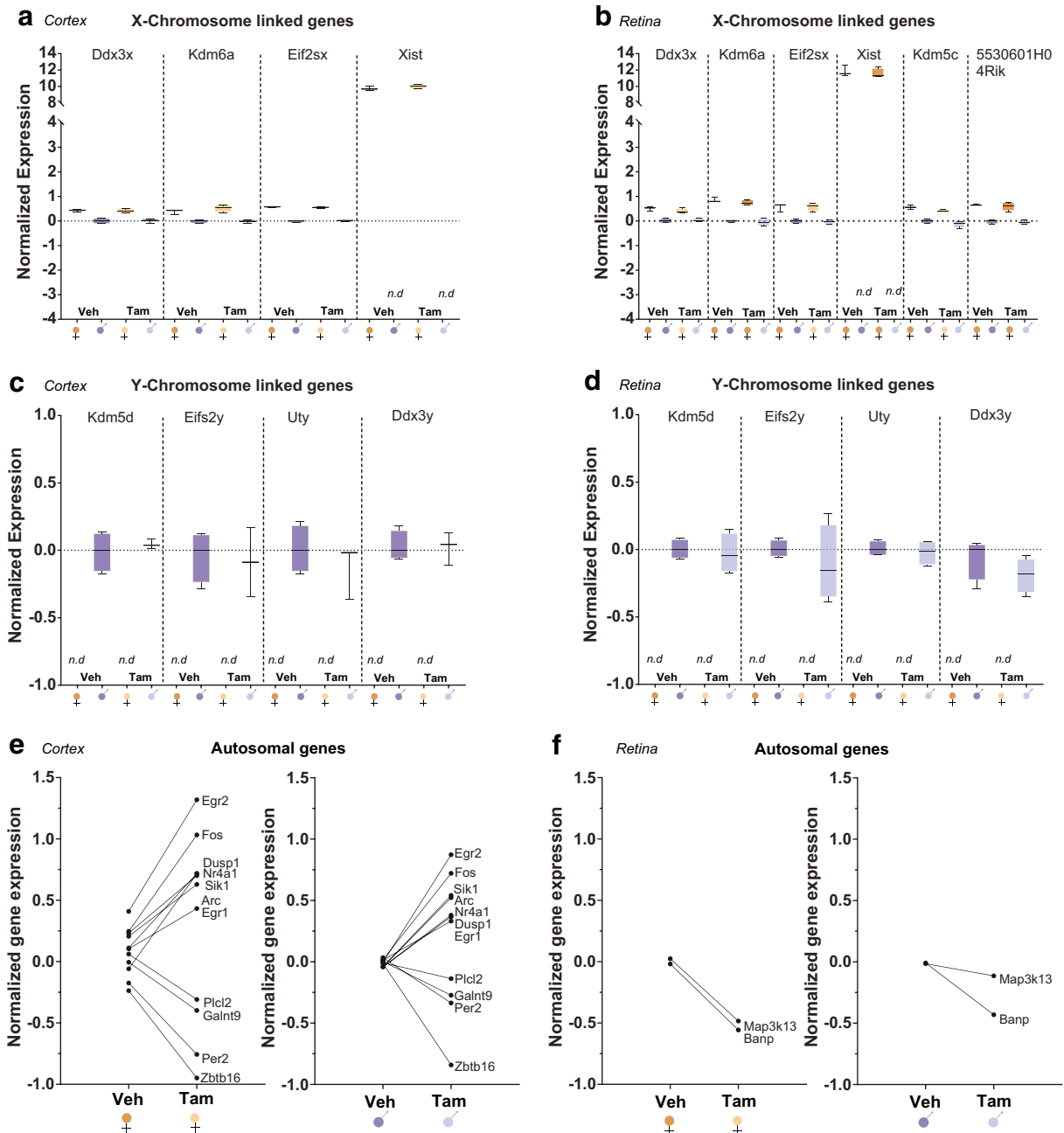


Fig. 4 Tam treatment does not introduce significant interactions effect with sex in retina or cortex. At 3 months of age, male and female C57BL/6 J mice ($n = 3-4$ /group) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. One month after cessation of Tam treatment RNA-Seq was performed. Both in cortex and retina Tam treatment did not alter known sex

differences in expression of X (**a, b**) or Y chromosome encoded genes (**c, d**). A few differences in gene expression were observed with TAM treatment in the cortex (**e**) and retina (**f**), but these were generally equivalent in males and females. RNA-Seq data presented as normalized gene expression as described in detail in the “**Methods**” section.

detected bands exhibited three different sizes that were summed within each sample for densitometric quantification (band 1: ~170 kDa, dimer bands 2: ~

64 kDa, and single/dimer bands 3: ~40 kDa) (Fig. 5d). The detected band size for β actin was ~42 kDa, as expected.

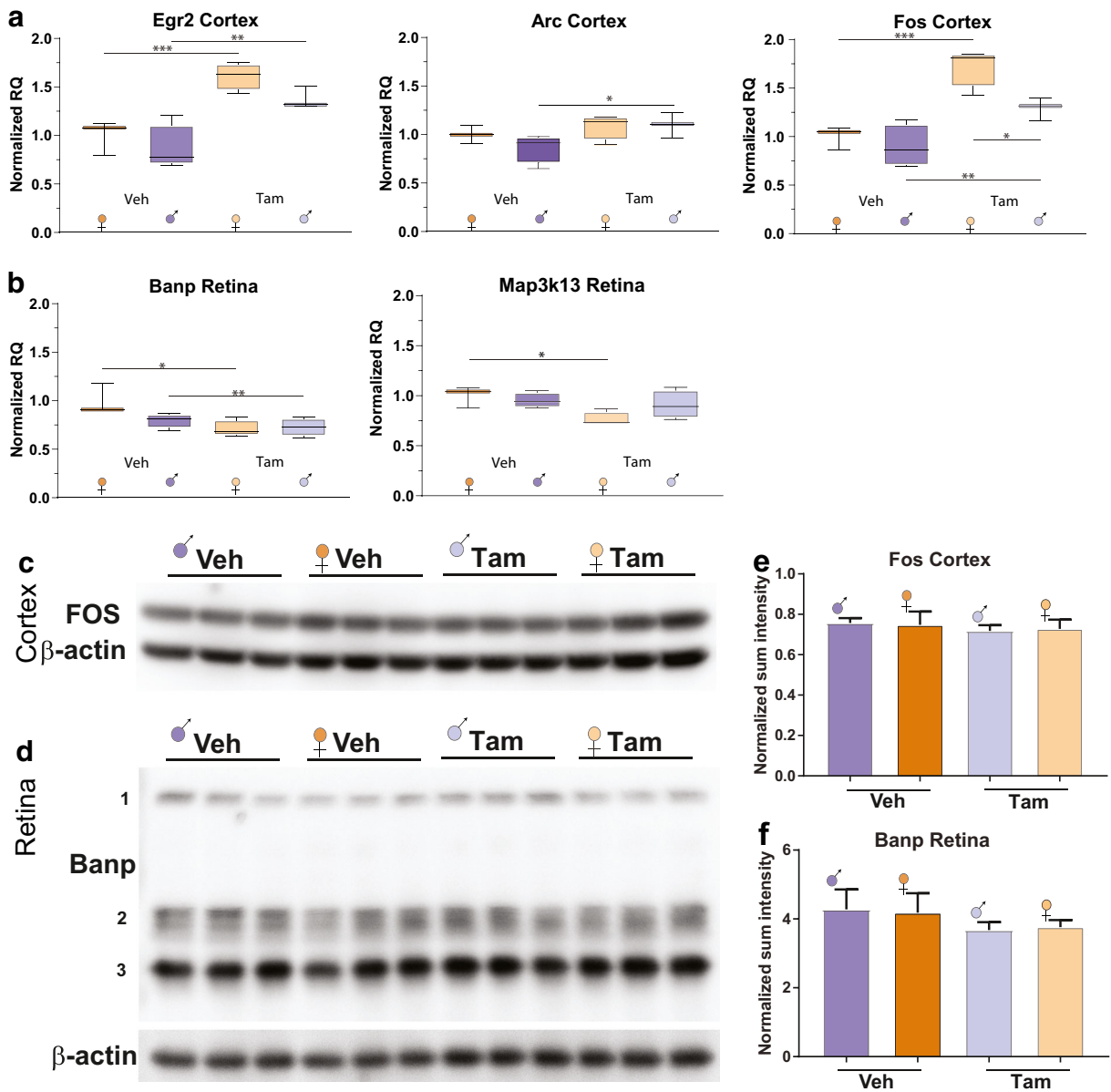


Fig. 5 Tam has minimal effect on the expression of selected autosomal transcripts across cortex and retina tissues. To confirm RNA-Seq findings qPCR was performed for selected gene targets. In the cortex, significant changes in Egr2, Arc, and Fos expression were evident (a). Only minimal interactions of sex with TAM treatment were evident, less than 25% differences. In the retina, very small differences with Tam treatment were observed for Banp and Map3k13. To further examine Tam effects, where specific antibodies were available, Fos and Banp protein expression in cortex (c) and retina (d) respectively were examined. No significant differences with Tam were observed. Box plots express

average normalized RQ for each group ($n = 3-4$) \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparing male and female groups under the same treatment by two-way ANOVA followed by all pairwise multiple comparison procedures (Holm-Sidak method). e–f The ratio of signal intensity of Fos and Banp bands relative to β -actin bands in cortex (e) and retina (f) by WB densitometry analysis is expressed as average normalized sum intensity for each group ($n = 3$) \pm SEM. Statistical analysis was performed by two-way ANOVA followed by all pairwise multiple comparison procedures (Holm-Sidak method)

The densitometry analysis revealed no significant differences between groups in the protein content of the protein tested in the cortex (Fig. 5e), or retina (Fig. 5f). In

conclusion, the minimal differences introduced by Tam at the transcript level were not recapitulated at the protein level across the cortex and retina tissues in our study.

TAM does not alter genome-wide methylation and hydroxymethylation

To our knowledge, there is no published control data on whether Tam administration affects DNA modifications. To assess levels of methylation (mC) and hydroxymethylation (hmC) in both CG and CH contexts whole-genome oxidative bisulfite sequencing was performed across sexes, treatments, and brain regions. Across all sexes, treatment groups, and brain regions equivalent methylation (~65%) were observed in the CG context (Fig. 6). Non-CpG methylation (mCH) was not altered by Tam treatment and was equivalent in males and females with higher levels in the cortex than hippocampus or retina. For hydroxymethylation in the CG context (hmCG), no effect of treatment of sex was evident with the highest hmCG levels being observed in the cortex followed by the hippocampus and then retina. No non-CpG hydroxymethylation was observed, in agreement with prior studies [(Hadad et al. 2016; Wen et al. 2014)].

TAM does not alter sex-chromosome methylation and hydroxymethylation

We have previously reported no age or sex differences in mCG-specific methylation levels in autosomes, but higher X chromosome methylation in females compared to males in hippocampus [(Hadad et al. 2016)]. To elucidate whether Tam could disrupt such sex differences, the methylation and hydroxymethylation patterns, specifically within X- and Y-chromosomes, were analyzed. Whole-genome oxidative bisulfite sequencing (WG-oxBS) data revealed male hippocampus had slightly and yet significantly lower mCG, while higher levels of mCH and hmCG in the X-chromosome compared to the female hippocampus (Fig. 7a). Such differential pattern of mCG, mCH, and hmCG in males and females was mirrored in cortex samples (Fig. 7B). Of interest, male retina samples had higher X-chromosome mCG, mCH, and hmCG levels than female retina (Fig. 7c). There was no effect of Tam on X-chromosome mCG, mCH, or hmCG, across the three CNS tissues. Analysis of male hippocampus, cortex, and retina showed Y-chromosome mCG, hmCG, and mCH were also unaffected by Tam-treatment (Fig. 8a–8c). In summary, the epigenetic data generated in this study

supports no interactions between Tam and the existing sex differences in the brain at the level of DNA modifications.

Discussion

Recently, significant contributions have been made in geroscience research that suggest potential pathways as targets for intervention to increase the lifespan and counteract frailty [(Ashpole et al. 2017; Deepa et al. 2017; Podlutzky et al. 2017)]. In this context, and taking into account the need for sex differences/dimorphisms to be accounted in animal research [(Masser et al. 2017; Clayton and Collins 2014; Ashpole et al. 2017)], well-controlled in vivo systems are a priority to avoid misinterpretation of data, specifically at the molecular level, as discussed below.

In light of the increasing interest in sex effects on the epigenome and transcriptome both in normal aging and disease settings, transgenic inducible cre mouse models that allow for manipulation of specific floxed genes, or tagging of cell-specific nuclei/and or polysomes represent valuable research tools. For instance, the cross of mice expressing cell-specific Tam-inducible cre recombinases with mice with floxed alleles, such as INTACT [(Mo et al. 2015)], TRAP [(Heiman et al. 2008)], RiboTag [(Sanz et al. 2009)], and NuTRAP [(Roh et al. 2017)], is a promising strategy for interrogating the sex- and age-dependent differences in the transcriptomes and/or epigenomes of specific cell types in the adult CNS. Despite the advantages that such technical advancements have to offer to the field of neuroscience, the potential effects of Tam on the epigenetic and transcriptional processes in the brain are a concern. This concern stems from the fact that Tam could exert agonist/antagonist actions on endogenous estrogen receptors [(Pfaffl et al. 2001; Tee et al. 2004; Kuiper et al. 1997; Bramlett and Burris 2002; Webb et al. 2003; Riggs and Hartmann 2003; Wardell et al. 2014)] in addition to control of *Cre-lox*-mediated recombination. Such SERM-associated activities might have implications at the molecular level and consequently on cognitive function. The aim of the present study was to examine the long-lasting effects of Tam on the existing sex differences in the transcriptome as well as in genomic methylation and hydroxymethylation patterns in the brain.

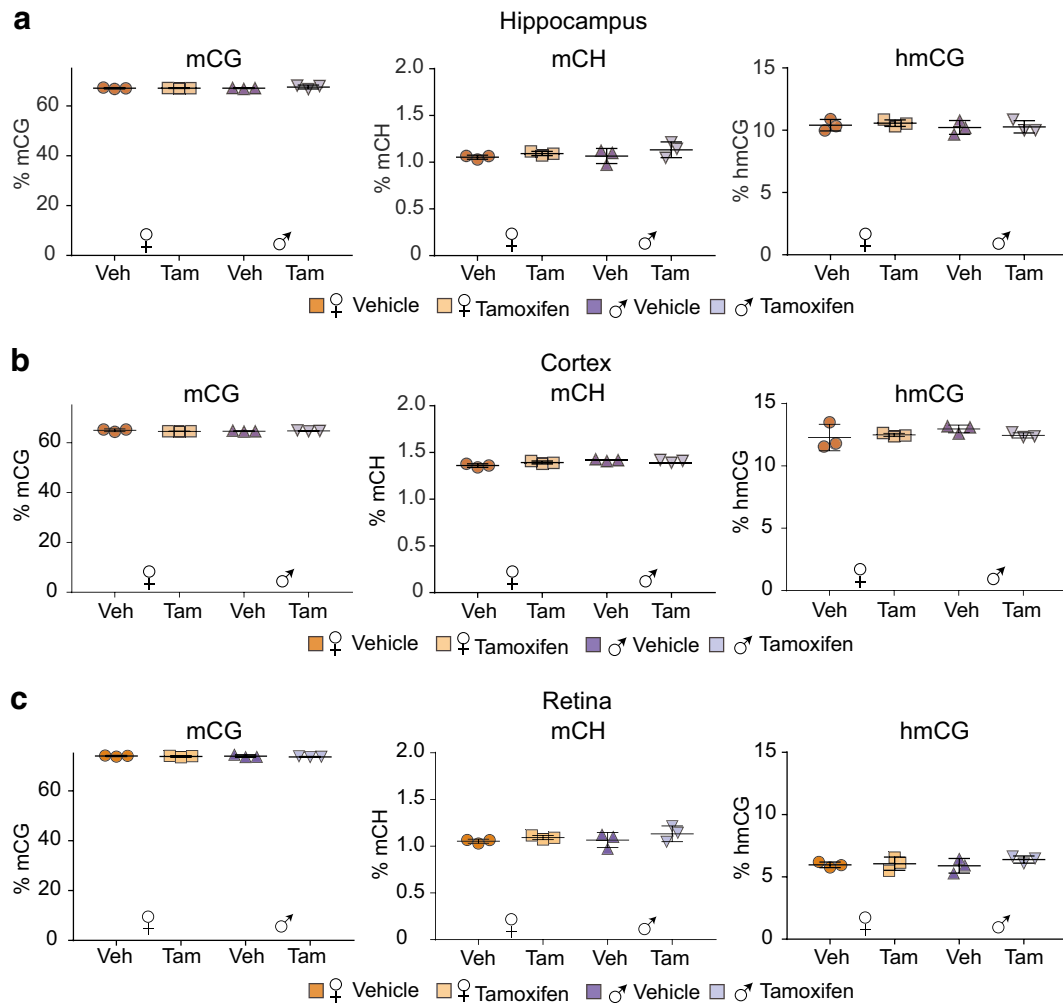


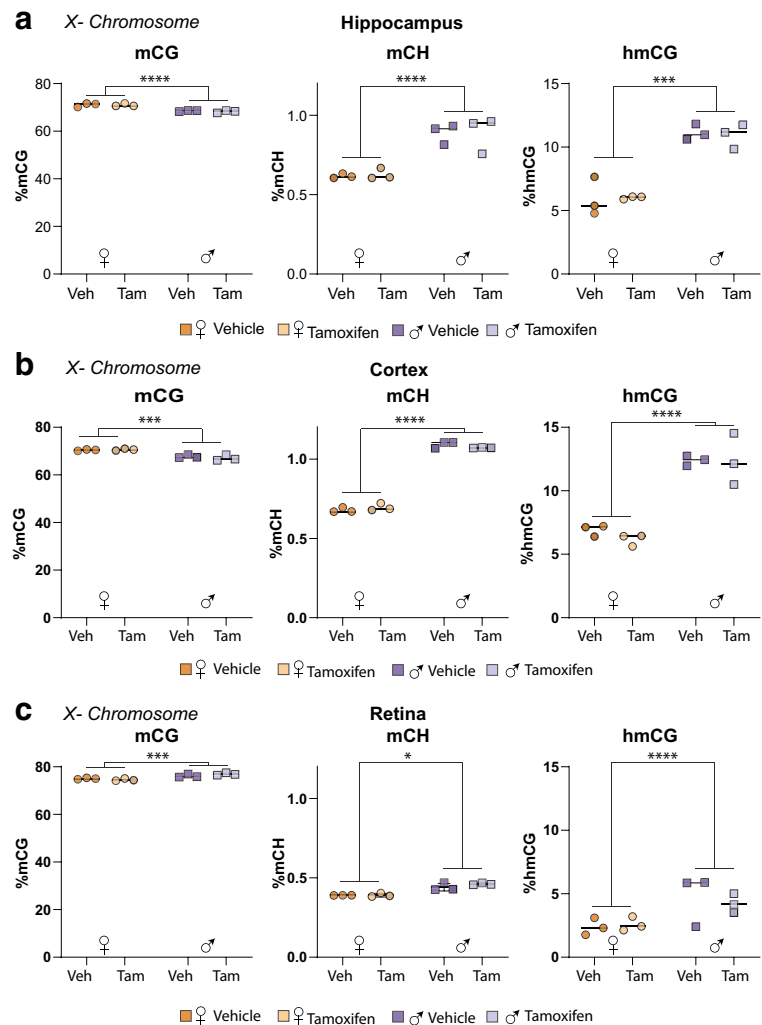
Fig. 6 Tam does not alter genome methylation and hydroxymethylation. At 3 months of age, male and female C57BL/6 J mice ($n = 3\text{--}4/\text{group}$) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. One month after cessation of treatment mC and hmC were analyzed in the hippocampus (**a**), cortex (**b**), and retina (**c**) by context (CpG and nonCpG (CH)) DNA isolated from tissues was subjected to

oxidative bisulfite conversion, library construction, and whole-genome oxidative bisulfite sequencing. No differences with treatment or sex in mCG, hmCG, or mCH were observed across all neural tissues assayed. hmCH was undetectable (not shown). Statistical analysis was performed by two-way ANOVA, with factors of treatment and sex, $p < 0.05$, Benjamini-Hochberg multiple testing correction

To address this question, we used C57BL/6 J male and female mice, subjected to a routinely used dose and regime of Tam i.p. injections to induce cre recombinases in a variety of tissues, including CNS [(Srinivasan et al. 2016; Jahn et al. 2018; Park et al. 2018)]. In the absence of cre expression, we were able to interrogate side effects of TAM on cytosine methylation and hydroxymethylation levels, in both CG and non-CG contexts and RNA-seq transcriptomic profiles of three commonly studied CNS tissues: hippocampus, cortex, and retina. Collectively, our findings support the Tam does not

affect existing sex differences, dimorphisms, and (presumably) divergences in the brain, epigenome- and transcriptome-wide. To our knowledge, this is the first report addressing potential side effects of this drug on the DNA modifications or at the entire transcriptome level. Here, we show that (1) Tam does not affect previously observed X-linked sex differences or Y-linked dimorphisms (Figs. 1 and 2), nor does it interact with sex differences in autosomal gene expression (Figs. 3, 4, and 5) in hippocampus, cortex, and retina; and (2) Tam does not alter genome-wide methylation and hydroxymethylation (Fig. 6) or

Fig. 7 Tam does not alter X-chromosome methylation and hydroxymethylation. At 3 months of age, male and female C57BL/6 J mice ($n = 3\text{--}4/\text{group}$) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. Following 1 month after cessation of treatment, their hippocampi (a), cortices (b), and retinae (c) were harvested and processed for oxidative bisulfite conversion, library construction, and whole-genome oxidative bisulfite sequencing. Sex differences were observed in X-chromosome mCG, mCH, and hmCG across all neural tissues assayed. No differences with in X-chromosome mCG, hmCG, or mCH were observed in any neural tissues assayed. Statistical analysis was performed by two-way ANOVA, with factors of treatment and sex, $p < 0.05$, Benjamini-Hochberg multiple testing correction

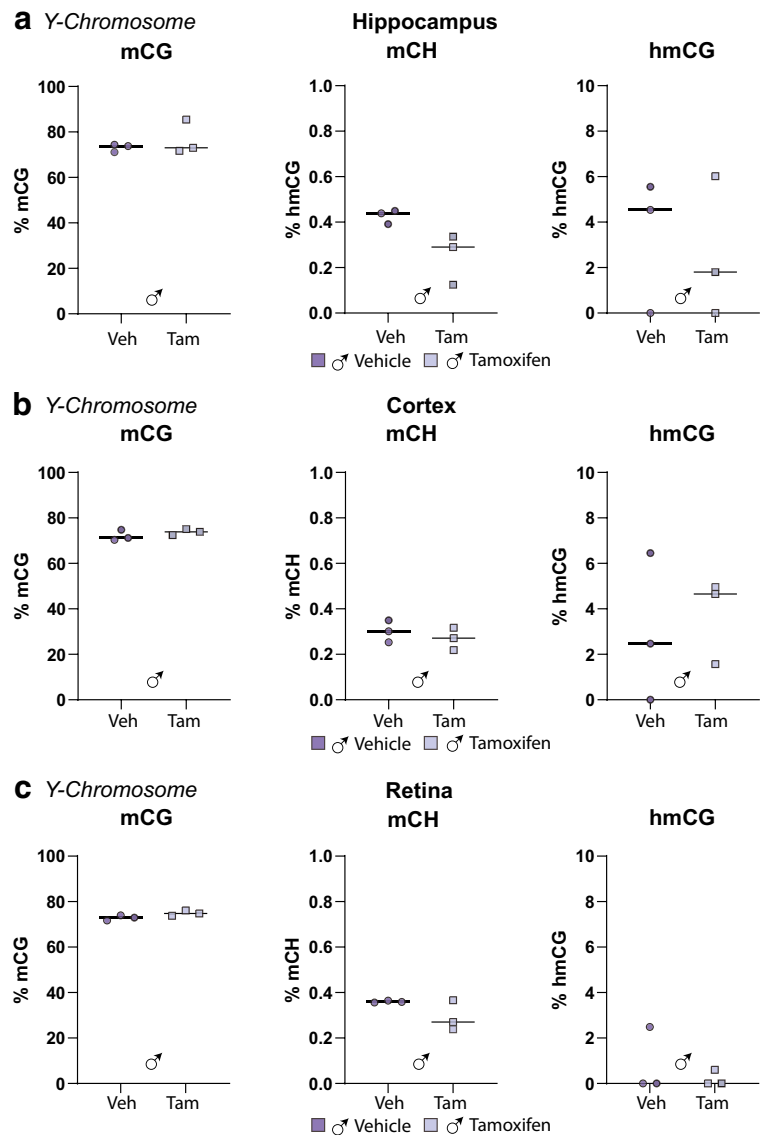


previously described sex-chromosome methylation and hydroxymethylation (Figs. 7 and 8) across tested CNS tissues. Importantly, Tam administration does not demonstrate lasting sex-specific effects either.

The toxicity of Tam as a confounding factor in experiments has been a matter of concern across research animal models, tissues, and with timing of induction [(Patel et al. 2017; Rotheneichner et al. 2017; Karlsson 2006; Morales-Otal et al. 2005)]. In testis—which responds to estrogen signaling and produces endocrine factors—adverse effects of Tam have been reported [(Patel et al. 2017; Karlsson 2006; Morales-Otal et al. 2005)]. A study by Patel et al. described long-term consequences of a single Tam injection on the testis of male mice at early pubertal ages (~p21), with

persistent adverse changes to both spermatogenic and endocrine functions of the testis that could be detected weeks later [(Patel et al. 2017)]. From those reported findings (Patel et al. 2017), it seems reasonable to infer that Tam induction for cre recombination at a borderline developmental time window with respect to full sexual maturation might have long-lasting effects that persist or even worsen with age and might affect almost every tissue in the body. In contrast, the experiments in this study, started at 3 months of age, and were analyzed at ~4 months of age, which led to no apparent dysfunctional phenotype, and most importantly to our purpose, no adverse effects of Tam on the sex-dependent differences in methylation and transcriptome signatures in the brain.

Fig. 8 Tam does not alter Y-chromosome methylation and hydroxymethylation. At 3 months of age, male and female C57BL/6 J mice ($n = 3\text{--}4/\text{group}$) were subjected to daily, ip delivery of Tam or Veh (control) for 5 consecutive days. Following 1 month after cessation of treatment, their hippocampi (a), cortices (b), and retinae (c) were harvested and processed for oxidative bisulfite conversion, library construction, and whole-genome oxidative bisulfite sequencing. No differences with in Y-chromosome mCG, hmCG, or mCH were observed in any neural tissues assayed. Statistical analysis was performed by two-way ANOVA, with factors of treatment and sex, $p < 0.05$, Benjamini-Hochberg multiple testing correction



Consistent with our experimental design and results, Rotheneichner et al. (Rotheneichner et al. 2017) showed neither the route of application, nor sex of treated mice influenced the response to Tam. Furthermore, Tam does not impact adult neurogenesis or behavioral performance [(Rotheneichner et al. 2017)].

In summary, no long-lasting effects of Tam treatment in the transcriptome or epigenome were found in any of the CNS tissues analyzed in our study. In agreement with recently reported data that Tam alone does not cause long-lasting changes in behavior and

neurogenesis, our findings support that Tam can be safely used as a cre-recombinase inducer without introducing significant confounds in transcriptomic and epigenomic studies in the adult CNS.

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