Title: Impact of X/Y genes and sex hormones on mouse neuroanatomy

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Abstract

Biological sex influences brain anatomy across many species. Sex differences in brain anatomy have classically been attributed to differences in sex chromosome complement (XX versus XY) and/or in levels of gonadal sex steroids released from ovaries and testes. Using the four core genotype (4CG) mouse model in which gonadal sex and sex chromosome complement are decoupled, we previously found that sex hormones and chromosomes influence the volume of distinct brain regions. However, recent studies suggest there may be more complex interactions between hormones and chromosomes, and that circulating steroids can compensate for and/or mask underlying chromosomal effects. Moreover, the impact of pre vs post-pubertal sex hormone exposure on this sex hormone/sex chromosome interplay is not well understood. Thus, we used whole brain high-resolution ex-vivo MRI of intact and pre-pubertally gonadectomized 4CG mice to investigate two questions: 1) Do circulating steroids mask sex differences in brain anatomy driven by sex chromosome complement? And 2) What is the contribution of pre- versus post-pubertal hormones to sex-hormone-dependent differences in brain anatomy? We found evidence of both cooperative and compensatory interactions between sex chromosomes and sex hormones in several brain regions, but the interaction effects were of low magnitude. Additionally, most brain regions affected by sex hormones were sensitive to both pre- and post-pubertal hormones. This data provides further insight into the biological origins of sex differences in brain anatomy.

Keywords: sex differences, sex hormones, sex chromosomes, mouse MRI, four core genotypes, compensation

Highlights

- Whole brain MRI is used to investigate how sex chromosomes (XX vs. XY) and pre- and post-pubertal gonadal hormones influence brain anatomy.
- Sex chromosomes and gonadal hormones have distinct effects on brain anatomy and can interact both cooperatively and competitively to shape brain structure.
- Both pre and post-pubertal hormones alter brain anatomy.
- Post-pubertal hormones are needed to establish or maintain many sex differences in brain anatomy.

Introduction

Biological sex affects brain structure and function (L. Cahill, 2006; Sacher et al., 2013; Tunç et al., 2016). In humans, male brains are on average 10% larger than female brains, and there are regional sex differences in volume and tissue density (Ruigrok et al., 2014). Sex also influences many brain functions, including memory, spatial navigation, pain perception, emotion, and mating and parenting behaviour (Andreano and L. Cahill, 2009; L. Cahill, 2006; Mogil, 2012). Understanding the origins of neurobiological sex differences is important, as they may underlie sex differences in prevalence, onset, and symptomatology of neuropsychiatric diseases. For instance, early-onset disorders such as autism and attention-deficit disorder are more common among males, while later-onset disorders such as depression and anxiety are more common among females (Bao and Swaab, 2010; Rutter et al., 2003).

The core biological causes of sex differences in the brain are classically defined as 1) activational effects of gonadal hormones; 2) organizational effects of gonadal hormones; and 3) effects of sex chromosomes (Arnold, 2014; 2009). Activational hormone effects are acute sex differences that depend on the presence of sex hormones; these effects disappear after adult gonadectomy. Conversely, organizational effects are permanent sex differences caused by hormones during development; such effects persist even after adult gonadectomy.

Beginning in the 1980s, Arnold and Breedlove argued that the classic model of activational, organizational, and sex chromosomal effects is too simplistic (Arnold and Breedlove, 1985; Juraska et al., 2013). Organizational hormonal effects were classically believed to occur only perinatally; however, it has since been shown that hormones can have permanent effects even later in adolescence (Ahmed et al., 2008; Schulz et al., 2009). Additionally, this classic model does not account for potential interactions between hormones and chromosomes (Arnold, 2014). Historically it was presumed that sex hormones and sex chromosomes always act to increase the extent of sex difference,

i.e. to make males more different from females or vice versa. However, studies now show that there may be both a) cooperative interactions between these factors, in which sex hormones and chromosomes have synergistic effects on a given phenotype, and b) compensatory effects, in which hormones and chromosomes compete or act in opposition, thereby reducing sex differences (Chen et al., 2012; Palaszynski et al., 2005).

A powerful tool for distinguishing the effects of sex hormones versus sex chromosomes is the “Four Core Genotype” (4CG) mouse model (De Vries et al., 2002) (Figure 1). In these mice, the testis-determining Sry gene is deleted from the Y chromosome, and an Sry transgene is inserted onto an autosome. This decouples gonadal sex (ovaries vs. testes) from sex chromosome complement (XX vs. XY), producing both XX and XY mice with either testes or ovaries (Arnold and Chen, 2009; Goodfellow and Lovell-Badge, 1993). The 4CG model has been used widely to dissect both neural and non-neural phenotypes (Chen et al., 2012; 2015; Gioiosa et al., 2008; Quinn et al., 2007; Seney et al., 2013). For instance, the 4CG model has been used to investigate the influence of sex chromosomes and hormones on a variety of behaviours, including spatial learning (Corre et al., 2014), habit formation, sex behaviours, nociception, and social behaviours (for review see Arnold and Chen, 2009). Likewise, we previously used high-resolution magnetic resonance imaging (MRI) and the 4CG model to demonstrate that while sex hormone effects are dominant in the brain, sex chromosomes influence a surprisingly large number of brain structures (Corre et al., 2014). We also showed that sex hormones and chromosomes usually affect separate brain areas (Corre et al., 2014). However, it is likely that the interplay is more complex and that either circulating hormones masked some effects of chromosomes on brain structure or that interactions between sex chromosomes and hormones were not fully delineated. Indeed, additional interactions between sex hormones and chromosomes have been revealed after gonadectomy in studies of other phenotypes (Arnold, 2014; Palaszynski et al., 2005).

Complete knowledge of the interactions between sex hormones and sex chromosomes is important as it will inform our understanding of the mechanisms that determine how sex differences in the brain are shaped and maintained. Here, we used whole-brain MRI and either intact or prepubertally gonadectomized 4CG mice to: 1) determine whether there are effects of sex chromosomes on neuroanatomy that are masked by circulating sex steroids, and 2) determine the relative contribution of pre- versus post-pubertal effects of sex hormones on neuroanatomy.

Figure 1: The Four Core Genotypes (4CG). The 4CG mouse model allows the dissociation of the effects of gonadal sex (ovaries vs. testes) from sex chromosome complement (XX vs XY). This is achieved via a genetic manipulation in which the *Sry* gene, which initiates testis development, is removed from its normal location on the Y chromosome and replaced with an *Sry* transgene inserted on chromosome 3. Consequently, testis development is no longer determined by the presence of a Y chromosome. Breeding wildtype XX females (XX F) with XY mice with this translocated *Sry* (XY M) produces the Four Core Genotypes: XY gonadal males (XY M) that develop testes, XX gonadal males that develop testes (XX M), XY gonadal females that develop ovaries (XY F), and XX gonadal females (XX F) that develop ovaries. Comparing mice with different gonads reveals the effects of sex hormones, while comparing mice with different chromosome complements reveals the effects of an XX or XY genome.

Materials & Methods

Mice:

All studies and procedures were approved by The Centre for Phenogenomics (TCP) Animal Care Committee (AUP 09-08-0175) in accordance with the recommendations of the Canadian Council on Animal Care, the requirements under Animals for Research Act, RSO 1980, and the TCP Committee Policies and Guidelines. The Four Core Genotype (4CG) mice used in this study were generated using B6.Cg-Tg(Sry)2Ei Sry^{dl1Rlb}/ArnoJ mice and purchased from Jackson labs (stock number 010905, RRID:SCR_002187)(De Vries et al., 2002). These mice possess two mutations: the *dl1Rlb* allele (henceforth referred to as Y⁻) is an 11kb deletion in the testes-determining/sex-determining (*Tdy* or *Sry*) region of the Y chromosome(Lovell-Badge and Robertson, 1990). Mice with the Y⁻ allele have a Y chromosome but develop as gonadal female with ovaries instead of testes. The second mutation is the insertion of the Tg(Sry)2Ei transgene (*Sry*) - a clone of the deleted *Sry* region - onto an autosome (Itoh et al., 2015; Mahadevaiah et al., 1998). Mice that possess this allele are phenotypically male and develop testes (Gubbay et al., 1990; Koopman et al., 1991). In the B6.Cg-Tg(Sry)2Ei Sry^{dl1Rlb}/ArnoJ mice, the two mutations are combined, which transfers testes determination from the Y chromosome to an autosome. Breeding these XY M mice with wildtype C57BL6/J XX females produces the Four Core Genotypes used in this study: XY⁻ *Sry* mice possess both the Y⁻ and *Sry* alleles and develop as gonadal males (XY M); XX *Sry* develop as gonadal males with testes (XX M); XY⁻ are gonadal females that lack the *Sry* gene and so develop ovaries (XY F); and XX females possess neither mutant allele so develop ovaries (XX F, Figure 1) (De Vries et al., 2002). Both XX F and XY F are gonadally female, and we previously showed that there is no difference in the pubertal timing or estrous cycle between these sets of animals (Corre et al., 2014).

C57BL6/J mice for breeding and experimentation were purchased from the in-house colony at TCP. Mouse genotypes from tail biopsies were determined using real-time PCR with specific probes designed for each allele (Transnetyx, Cordova, TN).

Experimental mice were maintained under controlled conditions (25°C, 12/12 hour light/dark circle) at TCP in individually ventilated, sterile cages. Mice were provided a standard irradiated chow from Harlan (Envigo (Harlan), Teklad Global 18% Protein Rodent Diet, 2018, 2826 Latham Drive, Madison WI 53713) and sterile water *ad libitum*.

Experimental design

At weaning, 4CG mice were randomly assigned to be intact controls or to undergo pre-pubertal gonadectomy. Two cohorts of mice were used in this study: the first cohort included the mice used in our previous study, all of which were gonadally intact (18 XX F, 16 XY F, 23 XX M, and 22 XY M) (Corre et al., 2014). A second cohort was added for this study and included both gonadectomized (21 XX F, 23 XY F, 18 XX M, and 20 XY M) and gonadally intact mice (8 XX F, 14 XY F, 6 XX M, and 9 XY M) to control for cohort effects between the two studies. Imaging, weight, and length data were acquired for all mice used in the study, from both cohorts. We also monitored pubertal timing and the estrous cycle of the mice in cohort 1 (Corre et al., 2014). A subset of mice from both cohorts (intact mice: 9 XX F, 11 XY F, 12 XX M, 15 XY M; gonadectomized: 10 XY F, 10 XY M) underwent behavioural phenotyping (Corre et al., 2014). A description of the behavioural training is described below. To account for these slight procedural variations, we covaried for whether behaviour testing was carried out in our analysis (along with covarying for experimental cohort). The total numbers of mice used in each group is outlined in table 1.

Table 1 - Number of mice used in the study

Genotype	Intact	Gonadectomized
XX F	26	21
XY F	30	23
XX M	29	18
XY M	31	20

Pre-pubertal gonadectomy:

4CG pups were bilaterally gonadectomized at weaning (postnatal day 21 +/- 1 day) under isoflurane anaesthesia (1-4%). This method was selected as previous studies show that even repeated exposure to isoflurane during development (up to 5-6 two hour exposures over 11 days) has very little impact on brain anatomy (Szulc et al., 2015). Ovariectomies were performed via dorsal incisions. The ovaries were removed after cauterization of each uterine horn below the ovary and fallopian tubes. Male castrations were performed via an abdominal incision. Each testicle was removed after cauterization of the vas deferens. The inner skin layer was closed using absorbable sutures and the outer skin layer was closed with staples. After the surgery the mice were housed individually for one week to allow wound healing. 2 mg/kg metacam and 0.5 ml saline was injected subcutaneously on the day of surgery and for two additional days for analgesia and hydration. Recovery was enhanced by feeding diet gel for two days. One week after surgery the wound clips were removed and mice were pooled so that, if possible, 3-4 mice were housed together. Males and females were housed separately.

Behavioural testing

A subset of mice were trained on a spatial version of the 8-arm radial arm maze (RAM) as previously described (Corre et al, 2014). The goal of this task is to learn the location of 4/8 arms that are baited with food rewards. To motivate mice to seek food rewards, mice that underwent RAM training were gradually food deprived. Food access was reduced to 1 hour over the course of 1 week. Mice had ad

libitum access to water throughout training and food deprivation, and were weighed daily to ensure they did not fall below 85% of their free-feeding weight.

Habituation & RAM training

The 8-arm radial arm maze (RAM) (MedAssociates, St. Albans, VT) consists of 8 identical, clear, plexiglass arms radiating from a central octagonal platform. Automatic pellet dispensers were placed at the end of each arm, which were blocked with a clear plastic door. Noldus Ethovision software was used to track the mouse's movements and to automatically control access to the arms and release of food reward from the pellet dispensers. Four spatial cues were placed distal to the maze to facilitate spatial learning, and did not move during training. Prior to RAM training, mice were habituated to the maze for three days. Food pellets were scattered throughout the maze and maze arms, and each mouse was given 8 minutes to explore. To prevent the mice from learning the spatial configuration of arms, a curtain surrounded the maze during habituation.

Mice were trained for 10 trials per day for 5 days. In each trial, an individual mouse was placed in the centre of the maze with all arms closed. After a delay of 5 seconds, the doors to all arms opened, and the mouse was given 8 minutes to explore the maze. Correct visits were defined as a mouse's first visit to a baited target arm. For a correct visit, the mouse received a food reward via the automated pellet dispenser when it reached the end of the arm; subsequent visits to target arms were not rewarded. After visiting an arm, the mouse returned to the centre of the maze. Between arm choices, the mouse was confined to the centre for 10 seconds to prevent it from visiting the arms serially. Once the mouse had visited all 4 baited arms or after 8 minutes, the trial ended, and the mouse was returned to its home cage. An individual mouse performed each of its 10 trials in succession, with an inter-trial interval of 90 seconds. After each trial, the maze was cleaned with Clidox to eliminate

olfactory cues. All behavioural tests were performed between 8:00 am and 5:00 pm. Mice were 58 +/- 4 days old on the first day of training.

Statistical analysis of behavioural tests

The percent correct visits out of total arm visits was defined as the number of correct visits out of the total arm visits times 100. Behavioural data was analyzed using the R statistical language and the lme4 (linear mixed effects models using Eigen and S4, <http://cran.r-project.org/web/packages/lme4/index.html>) and languageR libraries. Linear mixed effects models were used to test for interaction effects between gonadal sex (M vs F), gonadectomy (intact vs gonadectomized), and training day on each performance metric. As fixed effects, we used day of training (day), sex, gonadectomy, and sex:gonadectomy interaction, with random intercepts and slopes for each mouse. To determine the significance of a given effect, p-values were obtained by likelihood ratio tests of the full model with the effect in question against the model with that effect omitted.

Brain perfusion, fixation, and image acquisition:

Mice were perfusion-fixed on postnatal day 65 +/- 3 as previously described (L. S. Cahill et al., 2012; Lerch et al., 2011). Briefly, mice were placed in a supine position and perfused via the left cardiac ventricle using 30 ml of phosphate-buffered saline (PBS) (pH 7.4), 2 mM ProHance® (gadoteridol, Bracco Diagnostics Inc., Princeton, NJ), and 1 µl/ml heparin (1000 USP units/ml, Sandoz Canada Inc., Boucherville, QC) at room temperature (25°C) at a rate of approximately 60 ml/h. 30 ml of 4% paraformaldehyde (PFA) in PBS containing 2 mM ProHance® was subsequently infused at the same rate. After fixation, the heads were removed from the bodies, along with the skin, ears, and lower jaw. The skull was allowed to postfix in 4% PFA at 4°C for 12 h before being placed in a solution of PBS, 2 mM ProHance®, and 4°C 0.02% sodium azide (sodium trinitride, Fisher Scientific, Nepean, ON) until imaged 3-4 weeks postmortem.

Anatomical images of brains within skulls were acquired using a multi-channel 7.0-T scanner with a 40 cm diameter bore magnet (Varian Inc., Palo Alto, CA). A custom-built 16-coil solenoid array was used to image 16 samples concurrently (Dazai et al., 2011; Lerch et al., 2011). The MRI parameters used were optimized for gray-white matter contrast: a T2-weighted 3D fast spin-echo sequence, with TR = 2000 ms, echo train length = 6, TE_{eff} = 42 ms, field-of-view (FOV) = $25 \times 28 \times 14$ mm and matrix size = $450 \times 504 \times 250$. The resulting images had 56-micron isotropic resolution, and total imaging time was 11.7 hours. To correct for geometric distortions resulting from imaging in coils not located in the centre of the magnetic field, MR images of precision-machined phantoms were aligned towards a computed tomography (CT scan) of the same phantom. The resulting distortion correcting transformations were applied to all acquired images in a coil-specific manner.

Image Registration and calculation of brain structure volumes:

The brain images of all subjects in the study were aligned using an automated image registration pipeline as described previously (Lerch et al., 2011; 2008). Briefly, the images were first linearly aligned using a series of rotations, translations, scales, and shears and then locally deformed through an iterative nonlinear process (Friedel et al., 2014; Lerch et al., 2008). This pipeline brings all scans into precise anatomical alignment in an automated and unbiased fashion. All registrations were performed with a combination of mni_autoreg tools (Collins et al., 1994) and Advanced Normalization Tools (ANTS)(Avants et al., 2008). The output of the registration process is a consensus average that represents the average anatomy of all mice in the study, along with deformation fields that relate each individual image to the average. The deformation fields encode how each individual mouse differs from the study average and can be used to compare anatomy between the groups (Lerch et al., 2011; 2008). Specifically, the Jacobian determinant of each deformation field provides a measure of the volume of each voxel in the image. After registration, a pre-existing labelled MRI atlas that segments

the brain into 62 distinct regions was automatically aligned with the study average (Dorr et al., 2008). This atlas was further manually segmented to delineate the medial amygdala and medial preoptic area of the hypothalamus. The final atlas and Jacobian determinants were then used to calculate the volume of each of the labelled structures for each mouse in an automated fashion (Lerch et al., 2011; 2008). The software used for image registration is freely available here: <https://github.com/Mouse-Imaging-Centre/pydpiper> (v1.15). The sensitivity of the registration software has been evaluated in the manuscript by van Eede et al. (2013). The image registration was performed on the gnu supercomputer at the SciNet HPC Consortium (Loken et al., 2010). A script showing how the images were registered and volumes computed is included in the supplementary material (Insert Supplementary File here: [summary_of_analysis_for_neuroimage_2017.html](#)).

Image analysis:

All analyses were done using the R statistical language (R Core Team, 2016, <https://www.R-project.org>) using the RMINC library (<https://github.com/Mouse-Imaging-Centre/RMINC>). Error bars represent 95% confidence intervals throughout.

Two mechanistic questions were addressed in this study: 1) Does gonadectomy alter the effects of sex chromosomes on brain anatomy? and 2) What is the relative contribution of pre- versus post-pubertal sex hormone exposure to brain anatomy? For each of these questions, two main types of image analysis were performed: volumetric (structure-wise) analysis and voxel-wise analysis. For each of the 64 structures in the atlas, an ANOVA was performed to examine differences in brain structure volume due to interactions between gonadectomy status (intact vs. gonadectomized) and either sex chromosome complement (XX vs. XY) or sex hormones (gonadal male vs. gonadal female). The volumes of each of the 64 brain structures were normalized to total brain volume (structure volume/total brain volume * 100%) and therefore represent the percentage of brain volume. The volumes were

normalized for two reasons: first, XY brains were significantly smaller than XX brains (effect of sex chromosome complement ($F(1,192)=4.04, p=0.046$), and there was a trend towards increased brain volume in males (XX M and XY M, $F(1,192)=3.845, p=0.051$). Secondly, we have previously shown that most between-animal anatomical variability in mouse imaging studies is due to variations in total brain volume, rather than variation in the volume of specific local structures. Therefore using normalized volumes increases our power to detect subtle differences in anatomy due to hormones, chromosomes, or gonadectomy (Lerch et al., 2012).

We covaried for the factors Experimental Cohort (previous vs. current study), Training (trained vs. untrained), and either Sex or Chromosomes so that the final models used were:

$$1) \text{ Normalized Volume} = \beta_0 + \beta_1 \text{Cohort} + \beta_2 \text{Training} + \beta_3 \text{Sex} + \beta_4 \text{Gonadectomy} + \beta_5 \text{Chromosomes} + \beta_6 \text{Gonadectomy:Chromosomes} + \epsilon \text{ and}$$

$$2) \text{ Normalized Volume} = \beta_0 + \beta_1 \text{Cohort} + \beta_2 \text{Training} + \beta_3 \text{Chromosomes} + \beta_4 \text{Gonadectomy} + \beta_5 \text{Sex} + \beta_6 \text{Gonadectomy:Sex} + \epsilon$$

For the voxel-wise analysis, the same type of ANOVAs were performed at each voxel to test for interactions between gonadectomy and either sex chromosomes or sex hormones on local voxel volume. In these models, the log of the Jacobian determinant (which captures voxel volume) was used as the dependent variable and was adjusted to exclude volume differences due to differences in overall brain volume. These Jacobian determinants were calculated using the deformation fields that exclude overall brain volume differences. We refer to these relative Jacobian determinants as “relative voxel size” in the text and figures below. For both the volumetric and voxel-wise analysis, the False Discovery Rate (FDR) was used to control for false positives, and a 10% FDR was used as a threshold for statistical significance (Genovese et al., 2002). This threshold was chosen based on simulation studies showing that, at a 10% FDR, simulated volume losses/gains of 5-20% can be recovered with a

true false positive rate of $<0.2\%$ (van Eede et al., 2013). Moreover, this threshold reveals the extent of an effect within a given cluster of voxels, i.e. whether a volume difference is focal or diffuse. In addition to p-values, we report q-values, which are FDR-adjusted p-values.

In our voxel-wise analysis we also asked whether the magnitude of the effects of chromosomes or hormones differed in intact versus gonadectomized mice. At each voxel, the percent difference in voxel volume between intact XY and XX mice was calculated, excluding differences in overall brain size. Similarly, we calculated the percent difference in voxel volume between gonadectomized XY and XX mice. This procedure produced images that are referred to as “percent difference maps”. To determine whether there was a difference between the percent difference maps - i.e. whether the difference between XY and XX mice was larger or smaller in gonadectomized mice than in intact mice - we bootstrapped the probability that the absolute percent difference between intact XY and XX mice was larger than that between gonadectomized XY and XX mice. To do so, subjects (10 intact XY, 10 intact XX, 10 gonadectomized XY, and 10 gonadectomized XX) were randomly sampled with replacement from the dataset, and the absolute value of the percent difference between intact XY and XX mice and between gonadectomized XY and XX mice was computed at each voxel. Percent difference was defined as $(\text{XY voxel volume} - \text{XX voxel volume}) / \text{XX voxel volume} * 100$. This process was repeated 1000 times, and at each voxel, we calculated the frequency that the intact difference exceeded the gonadectomized difference. In the resulting probability maps, a probability greater than 0.75 indicates a greater than 75% chance that the volume difference between XY and XX mice is larger in intact mice than in gonadectomized mice. A probability less than 0.25 indicates a less than 25% chance the volume difference is larger in intact mice – in other words, a 75% chance that the effect is larger in the gonadectomized cohort. The same type of percent difference and probability maps

were also produced to compare the difference between intact gonadal males and gonadal females to that between gonadectomized males and females.

Results

Gonadectomy subtly alters impact of sex chromosomes on brain anatomy

We first examined the brain regions influenced by sex chromosome complement (XY vs. XX). It is important to note that in all analyses, XY mice include both XY F and XY M animals; likewise XX mice include XX F and XY M animals. To control for differences in sex steroids, we covaried for gonadal sex (see experimental methods). Sex chromosome complement significantly affected the volume of 27 brain structures, and the voxel-wide effects of sex chromosome complement on brain anatomy were wide-spread (Figure 2, see also Corre et al., 2014). Figure 2A displays a representative subset of these effects and depicts the percent difference in voxel size between intact XY and XX mice.

We then asked whether the sex chromosome-dependent differences observed in intact mice were modulated when post-pubertal sex hormones were eliminated via gonadectomy. The percent difference in voxel size between gonadectomized XY and XX mice is shown in Fig. 2B. In the examples shown in panels A and B, the percent difference maps were masked so that only voxels where there is a significant main effect of chromosomes on volume are shown. Qualitatively, the global pattern of chromosomal effects on brain anatomy appears quite similar in intact and gonadectomized mice, suggesting that any effect of gonadectomy on chromosome-dependent differences is of low magnitude.

However, to examine the interaction between sex chromosome and sex hormone effects quantitatively, we next asked whether gonadectomy revealed effects of sex chromosomes that were not observed in intact 4CG mice; that is, were there chromosomal effects that had been masked by the sex steroid milieu? Although the percent difference maps in Figure 2 appear coarsely similar, there were

percent difference
between XY & XX

some

Figure 2 (previous page): Sex chromosome-dependent effects on brain anatomy in intact and gonadectomized four core genotype mice. Coronal slices depict the percent difference in voxel size between intact XY and XX mice (A) or between gonadectomized (gdx) XY and XX mice (B) overlaid on the average anatomical MRI. The particular coronal slices shown were chosen to display representative brain areas in which sex chromosomes influence voxel volume. Only voxels in which there was a significant main effect of chromosomes on relative voxel size (voxel volume adjusted for total brain volume) at a false discovery rate (FDR) of 10% are shown. White lines point to illustrative voxels of interest whose relative voxel size is plotted in panel C. Overall, chromosomal effects are of similar extent in gonadectomized and intact mice, and there was no interaction between gonadectomy and sex chromosome complement at 10% FDR. However, at a more exploratory threshold of 15% FDR, gonadectomy did lead to subtle alterations to the effects of chromosomes in some voxels. Areas in which there was an interaction between sex chromosomes and gonadectomy at 15% FDR are outlined in white. CB: cerebellum SC: superior colliculus cc: corpus callosum HY: hypothalamus BNST: bed nucleus of stria terminalis Cpu: caudoputamen (striatum) ac: anterior commissure. gdx: gonadectomized.

local regions such as the corpus callosum, anterior commissure, and BNST where the effect of chromosomes appeared to be modulated by gonadectomy. Indeed, within the whole brain we identified

18 structures out of 64 delineated in our atlas where there was a significant interaction between sex chromosome complement and gonadectomy on normalized volume at 10% FDR. The most striking interaction effects were observed in the anterior commissure pars anterior and pars posterior, bed nucleus of the stria terminalis (BNST), inferior cerebellar peduncle, corpus callosum, and internal capsule (Table 2). With the exception of the inferior and middle cerebellar peduncles, cerebellar cortex, internal capsule, and mammillary bodies, the interaction between chromosome complement and gonadectomy remained significant even if absolute (non-normalized) volumes were used for the analysis.

Both attenuated and novel chromosomal effects emerged after gonadectomy. For example, in intact mice, the corpus callosum was larger in XX mice than in XY mice, independent of gonadal sex. This sex chromosome-dependent difference was significantly reduced after gonadectomy. Similarly, intact XY mice had larger relative inferior cerebellar peduncle volume than XX mice, but this difference between XX and XY mice was reduced after gonadectomy. These two examples demonstrate cooperative effects between sex chromosomes and sex hormones, in which sex hormones interact with

Table 2: Brain structures where there is significant interaction between sex chromosome complement and gonadectomy on normalized volume:

Structure	F-statistic (interaction)	Q-value*	Effect in Intact Mice (% difference between XY and XX +/- sd)	Effect in GDX Mice (% difference between XY and XX +/- sd)	Description
corpus callosum	9.5	0.038	XY < XX (-1.72% +/- 1.11%)	XY = XX (0.86% +/- 1.18%)	cooperation
fourth ventricle	9.82	0.038	XY > XX (5.11% +/- 2.86%)	XY = XX (-0.14% +/- 2.15%)	cooperation
habenular commissure	8.13	0.052	XY > XX (8.35% +/- 6.55%)	XY < XX (-1.00% +/- 5.18%)	cooperation
inferior cerebellar peduncle	7.91	0.052	XY > XX (5.11% +/- 2.12%)	XY > XX (1.73% +/- 1.62%)	cooperation
lateral septum	7.37	0.052	XY < XX (-2.16% +/- 1.31%)	XY = XX (0.88% +/- 0.95%)	cooperation
ventral tegmental decussation	7	0.052	XY < XX (-2.86% +/- 2.29%)	XY = XX (0.73% +/- 1.86%)	cooperation
thalamus	6.96	0.052	XY < XX (-2.01% +/- 1.26%)	XX = XY (-0.69% +/- 0.98%)	cooperation
mammillary bodies	6.5	0.057	XY < XX (-4.20% +/- 4.09%)	XX < XY (-2.02% +/- 2.89%)	cooperation
superior olivary complex	6.49	0.057	XY > XX (6.10% +/- 4.81%)	XY < XX (-1.42% +/- 3.09%)	cooperation
middle cerebellar peduncle	6.1	0.063	XY > XX (2.27% +/- 2.58%)	XY < XX (-1.72% +/- 1.91%)	cooperation
nucleus accumbens	5.93	0.063	XY < XX (-2.06% +/- 1.6%)	XY = XX (0.49% +/- 0.93%)	cooperation
BNST	16.28	0.005	XY = XX (-0.46% +/- 1.82%)	XY > XX (4.32% +/- 1.37%)	compensation
striatum	10.51	0.038	XY = XX (-0.76% +/- 1.13%)	XY > XX (1.09% +/- 0.95%)	compensation
globus pallidus	7.17	0.052	XY = XX (0.66% +/- 1.17%)	XY > XX (2.65% +/- 1.07%)	compensation
anterior commissure: pars anterior	5.99	0.063	XY = XX (0.93% +/- 1.53%)	XY > XX (4.00% +/- 1.32%)	compensation
internal capsule	5.62	0.071	XY = XX (0.83% +/- 1.14%)	XY > XX (2.04% +/- 0.90%)	compensation
anterior commissure: pars posterior	5.39	0.076	XY = XX (0.41% +/- 1.60%)	XY > XX (2.28% +/- 1.50%)	compensation
cerebellar cortex	5.15	0.082	XY = XX (-0.54% +/- 1.33%)	XY < XX (-2.22% +/- 1.00%)	compensation

*Q-value, FDR-corrected p-value. Compensation refers to effects in which sex hormones compensate for or mask chromosomal differences, and so new chromosomal effects emerge after gonadectomy. Cooperation refers to effects in which sex hormones and chromosomes act synergistically, and so chromosome-dependent effects are attenuated after gonadectomy. If the percent difference between XY and XX was less than 1%, the effect was listed as “XY=XX”.

sex chromosomes to enhance their effects. Conversely, in the anterior commissure, BNST, and internal capsule, there was no effect of chromosomes on volume in intact mice; however, after gonadectomy, these structures were larger in XY versus XX mice, regardless of gonadal sex. This provides evidence of compensatory effects of steroids, wherein sex hormones mask an underlying sex chromosome-dependent difference which is only observed when the hormones are removed.

The data in table 2 reflect structure-wise analyses. When similar analyses were performed at every voxel, no significant interactions were observed between sex chromosome complement and gonadectomy at 10% FDR; however, at slightly higher FDR thresholds of 10.8-14%, the voxel-wise results recapitulated the structure-wise interaction effects. This difference between the structure-wise and voxel-wise results may be because the size of change at each voxel is small, making the effects harder to detect. Indeed, within voxels where there was an effect of chromosome complement on volume, the absolute percent difference between XY and XX mice was on average reduced by 8% in gonadectomized animals. Thus, the modulating effects of gonadectomy on sex-chromosome dependent differences is quite subtle.

To further evaluate how gonadectomy modulates the effects of sex chromosomes on the brain, we next directly compared the percent difference in voxel volume between intact XY vs. XX mice and gonadectomized XY vs. XX mice. To do so, we bootstrapped the probability that the percent difference in voxel volume between intact XY and XX mice was larger than that in gonadectomized XY and XX mice (Figure 3A, probSex_mask.nii here). This analysis demonstrated a high probability that the effect of chromosomes is attenuated after gonadectomy in voxels in the corpus callosum, thalamus, superior olive, and ventral striatum, and that the effect of chromosomes is larger after gonadectomy in voxels in the anterior commissure, dorsal striatum, BNST, hypothalamus, globus pallidus, and internal capsule

Figure 3 (previous page): Comparison of percent difference maps in intact and gonadectomized mice. A) Comparison of sex chromosome effects. At each voxel, the percent difference between intact XY and XX mice was compared to that between gonadectomized XY and XX mice. To do so, the probability that the intact percent difference was greater than the gonadectomized difference was computed via bootstrapping. Coronal slices depict regions where the bootstrapped probability for a difference in the percent difference maps exceeded 75%. A probability greater than 0.75 indicates a greater than 75% chance that the percent difference is larger in intact mice than in gonadectomized mice, and thereby reduced with gonadectomy. A probability less than 0.25 indicates a less than 25% chance that the percent difference is larger in intact mice than in gonadectomized mice, i.e. a 75% chance the difference is larger after gonadectomy. Red areas indicate those where the effect of chromosomes was larger in intact mice than in gonadectomized mice. Blue areas depict those where the chromosome effect was smaller in intact mice, indicating that gonadectomy enhances the sex difference. Gonadectomy alters the effect of chromosomes in the corpus callosum, septum, striatum, anterior commissure, BNST, and hypothalamus. B) Comparison of hormone effects in intact versus gonadectomized mice. Voxels where the effect of sex hormones was larger in intact mice than in gonadectomized mice are depicted in red; those where the effect was smaller are shown in blue. Gonadectomy attenuates the effect of hormones in several canonically sexually dimorphic brain regions, including the medial amygdala and medial preoptic area of the hypothalamus. CB: cerebellum SC: superior colliculus SSp: primary somatosensory cortex cc: corpus callosum MeA: medial amygdala HY: hypothalamus CPu: caudate/putamen (striatum) BNST: bed nucleus of stria terminalis SEP: septum ac: anterior commissure MPOA: medial preoptic area. The full probability maps are available here: probSex_mask.nii and probXY_mask.nii.

(Figure 3A).

There were voxels in the septum that were both attenuated and larger after gonadectomy, but over the whole structure, the effect was attenuation. Taken together, our combined findings suggest that gonadectomy does alter the impact of sex chromosomes on several brain structures, but the effects are small in magnitude.

Pre- and post-pubertal hormones both impact brain anatomy

The 4CG model also allowed us to assess the effects of sex hormones independent of sex chromosome complement. Consistent with both our prior results and previous literature, sex hormones significantly affected the volume of many of the canonically dimorphic brain regions, including the medial preoptic area of the hypothalamus (mPOA), BNST, hypothalamus, and medial amygdala (Figure 4)(L. Cahill, 2006; Corre et al., 2014; Juraska et al., 2013; McCarthy et al., 2015; Schwarz, 2016). Moreover, pre-pubertal gonadectomy also enabled us to assess whether sex hormone effects on brain anatomy are due to hormone exposure early (in utero and pre-pubertal) or late (pubertal and post-

Figure 4 (previous page): Sex hormone-dependent effects on brain anatomy in intact and gonadectomized four core genotype mice. Coronal slices depict the percent difference in voxel size between intact gonadal male and gonadal female mice (A) or between gonadectomized male and female mice (B) overlaid on the average anatomical MRI. The particular coronal slices shown were chosen to display representative brain areas in which sex chromosomes influence voxel volume. Only voxels in which there was a significant main effect of sex hormones or an interaction between hormones and gonadectomy on relative voxel size (voxel volume adjusted for total brain volume) at 10% FDR are shown). Areas in which there was an interaction between hormones and gonadectomy at 15% FDR are outlined in white, though almost all interactions had FDR-adjusted p-values less than 0.1. White lines point to individual, illustrative voxels of interest whose relative voxel size is plotted in panel C. Gonadectomy reduces hormone-dependent sex differences in many brain structures. CB: cerebellum SC: superior colliculus Ssp: primary somatosensory cortex MeA: medial amygdala BNST: bed nucleus of stria terminalis MO: motor cortex MPOA: medial preoptic area of the hypothalamus. Female: XX F or XY F. Male: XX M or XY M. gdx: gonadectomized.

pubertal) in development. In the mPOA and medial amygdala, there was a significant interaction between gonadal sex and gonadectomy on normalized structure volume (Table 3). The same interaction effects were observed when absolute (non-normalized) volumes were used. In the BNST and hypothalamus, although there was a reduction in the effect of sex hormones, the interaction between hormones and gonadectomy was not significant at 10% FDR; the associated q-values (FDR adjusted p-value) were $q=0.17$. Below, we further describe the effect of gonadectomy on these canonically dimorphic regions, and then present the influence of pre versus post-pubertal hormones across the whole mouse brain.

Table 3: Effect of pre- versus post-pubertal sex hormones on normalized brain structure volumes

Structure	F-statistic (interaction term)	Q-value*	Percent difference between intact males and females	Percent difference between gonadectomized males and females	Sex hormone effect attributable to pre-pubertal versus pubertal/post-pubertal effects
mPOA	11.22	0.03	3.33% +/- 1.84% (M > F)	-0.10% +/- 1.55% (M=F)	Established/maintained pubertally or post-pubertally
medial amygdala	15.63	0.01	6.36% +/- 1.75% (M > F)	2.18% +/- 1.38% (M > F)	Both pre- and post-pubertal
BNST	6.70	0.17	5.12% +/- 1.61% (M > F)	2.15% +/- 1.54% (M > F)	Both pre- and post-pubertal
hypothalamus	6.80	0.17	1.06% +/- 1.26% (M > F)	-0.13% +/- 0.94% (M=F)	Established/maintained pubertally or post-pubertally

*Q-value, FDR-corrected p-value

Post-pubertal hormones are required for sex differences in the mPOA

The most striking sex/gonadectomy interaction was in the mPOA (Figure 4 & 5). In intact mice, the normalized volume of the mPOA is 3.3% (+/- 1.8%) larger in gonadal males than in females (effect of sex: $F(1,111)=23.51, q=0.00009$). This effect was nearly eliminated in gonadectomized mice (interaction between sex and gonadectomy ($F(1,191)=11.23, q=0.03$) (Table 3, Figure 4 & 5).

Figure 5: Effect of sex hormones and sex chromosomes on the volume of the medial preoptic area and medial amygdala. Medial preoptic area (MPOA): In intact mice, the mPOA is significantly larger in gonadal males than gonadal females, regardless of sex chromosome complement. This sex difference is eliminated in pre-pubertally gonadectomized mice, indicating pubertal or ambient hormones are required to generate or maintain this sex difference. Medial amygdala (MeA): In intact mice, sex hormones and chromosomes exert opposing effects on MeA volume. MeA volume is significantly larger in gonadal males than gonadal females, but significantly smaller in XY mice versus XX mice. Gonadectomy significantly reduces the difference between males and females, but also reduces the effect of chromosomes on MeA volume.

Sex chromosomes and hormones exert opposing effects on medial amygdala volume

Like the mPOA, the medial amygdala is known to be larger in intact males than females. We found that this sex difference arises despite opposing effects of sex hormones and sex chromosomes (Figure 5). In intact mice, normalized medial amygdala volume was 6.36% (+/- 1.75%) larger in gonadal males versus females (Effect of gonadal sex: $F(1,111)=79.84$, $q<0.0001$), but 3.4% (+/- 2.18%) smaller in males and females with an XY chromosome complement than those with two X chromosomes (effect of chromosomes: $F(1,111)=24.6$, $q<0.0001$).

This sex difference in medial amygdala volume between gonadal males and females was still present in gonadectomized mice (Figure 4 & 5); however, the effect of sex hormones was attenuated by 66%, from a 6.36% (+/- 1.75%) difference between gonadal males and females to a 2.18% (+/- 1.38%) difference (sex-gonadectomy interaction: $F(1,191)=15.63$, $q=0.007$) (Table 3). After gonadectomy, the medial amygdala remained significantly larger in males than in females ($p=0.05$); however, it was significantly smaller in gonadectomized males compared to intact males ($p=0.00003$). Likewise, in our voxelwise analysis, there was a high (>0.75) probability that the difference between gonadal males and females was larger in intact mice than in gonadectomized mice (Figure 3B). Intriguingly, gonadectomy also reduced the difference in volume between XX and XY mice (Figure 5).

Pre- and post-pubertal hormones and sex chromosomes all influence BNST volume

The BNST is another canonically sexually dimorphic brain region that is larger in intact males than females (del Abril et al., 1987; Hines et al., 1992). This sex hormone-dependent difference was attenuated but not eliminated in pre-pubertally gonadectomized mice, demonstrating that both pre- and post-pubertal hormones affect the volume of this structure (Figure 6A). Gonadectomy did tend to reduce the difference in BNST volume between gonadal males and females from 5.1% (+/- 1.6%) to

Figure 6: Effect of sex hormones, sex chromosomes, and pre-pubertal gonadectomy on volume of the bed nucleus of the stria terminalis (BNST). A) In intact mice, BNST volume is larger gonadal in males than females. This effect is significantly reduced in pre-pubertally gonadectomized mice. B) When partitioned by sex chromosomes (XX vs XY), independent of gonadal sex, gonadectomy reveals an effect of sex chromosomes on BNST volume that is not present in intact mice. C) Analysis of the same data by genotype indicates that chromosomes and hormones interact to shape BNST volume. In intact mice, the BNST is larger in males regardless of sex chromosome complement. Gonadectomy specifically reduces the volume of both XX females and XX males, though XX males are most affected. In XX mice, the lack of pubertal or ambient hormones reduces BNST volume; however these hormones are not necessary for normal BNST volume in XY mice.

2.2% (+/- 1.5%), although only at an FDR of 17% (Figure 6A, Table 3). However, bootstrap analysis corroborates the finding that the sex difference (gonadal M vs F) was larger in intact mice than in gonadectomized mice (Figure 3B).

Surprisingly, gonadectomy also revealed novel, underlying chromosomal effects on the BNST.

In intact mice, the normalized BNST volume is 5.1% (+/- 1.6%) larger in intact males than females,

regardless of chromosome complement (Figure 6A & B). However, in gonadectomized mice, normalized BNST volume was 4.3% ($\pm 1.4\%$) larger in XY versus XX mice, regardless of gonadal sex (Figure 6B, interaction between chromosomes and gonadectomy: $F(1,191)=16.28, q=0.005$). This effect appears to be driven by a decreased volume in XX mice after gonadectomy (Figure 6C). This finding was recapitulated in the voxel-wise analysis, which showed a high probability that the difference between XX and XY mice is larger in gonadectomized mice than in intact animals (Figure 3A).

Interestingly, when we analyzed the volumes by genotype, we found that the reduction in the effect of sex hormones in gonadectomized males was mediated entirely by XX mice (Figure 6C). Indeed, gonadectomy reduced the volume of the BNST in XX males but not XY males. This implies that pubertal/post-pubertal hormones are not necessary for normal BNST volume in XY mice. The BNST volume was also reduced after gonadectomy in XX females but not XY females, though the effect was not dramatic as in XX males. This too suggests a complex interaction between sex chromosomes and sex hormones on BNST structure.

Pubertal and post-pubertal sex hormones influence non-canonically dimorphic brain areas

In addition to characterizing the influence of pre- versus post-pubertal hormones on canonically dimorphic brain areas, our whole-brain analysis revealed the impact of gonadectomy across the brain. Significant interactions between sex hormones and gonadectomy were observed in the somatosensory cortex, superior colliculus, and motor cortex (Figure 4). These areas were larger in intact gonadal females than males, but this effect was attenuated after pre-pubertal gonadectomy (Figure 3B). This indicates that post-pubertal hormones are needed to establish or maintain these sex differences.

Overall, within all voxels in the brain in which there was a significant effect of sex or significant sex-gonadectomy interaction, the mean percent difference between intact males and females was 4.6% (standard deviation=1.85%). In gonadectomized mice, this mean percent difference between males and females was markedly attenuated by approximately 59% to 1.91 % (standard deviation=1.22%). This attenuation implies that in these regions approximately 40% of the effects of sex hormones can be attributed to pre-pubertal hormone effects, while approximately 60% results from pubertal or post-pubertal hormone effects.

Pre-pubertal hormones influence spatial learning ability in the radial arm maze

Finally, we examined the impact of gonadectomy on a sex-hormone-dependent spatial learning behaviour, radial arm maze learning. Previously, we found that gonadal males learn the radial arm maze faster and make fewer errors than females, regardless of sex chromosome complement (Corre et al., 2014). Here, we asked whether this behavioural difference was due to pre- or post-pubertal sex hormones. Again, we found that in both intact and gonadectomized mice, gonadal males learned better than gonadal females (interaction between gonadal sex and training day: $X^2(1)=2.2, p=0.047$). There was no significant interaction between gonadectomy, gonadal sex, and day of training which indicates that spatial learning in the radial arm maze depends on pre-pubertal hormone effects.

Discussion

A critical question is the role of sex chromosomes versus sex steroids in modifying brain structure and function. Using the 4CG model, we previously found that sex chromosomes and hormones influence neuroanatomy but that few regions appeared to be influenced by both factors (Corre et al., 2014). As with other phenotypes, we hypothesized that there may be additional areas that

are influenced by sex chromosomes but where effects of circulating hormones mask underlying chromosomal effects (Palaszynski et al., 2005). Therefore, we investigated interactions between sex hormones and chromosomes on the anatomy of the whole brain, using intact and gonadectomized 4CG mice.

Pre-pubertal gonadectomy could alter the effects of sex chromosomes in the following ways:

1. Gonadectomy could reveal new chromosome-dependent structural differences that are not apparent in intact mice. This would indicate that hormonal effects mask or compensate for the impact of sex chromosomes.
2. Chromosomal effects observed in intact mice could be enhanced in gonadectomized mice, indicating that usual levels of sex hormones attenuate, or compensate for, these chromosomal effects on brain structure. Interaction types 1 and 2 between hormones and chromosomes have been referred to as "compensation" (Arnold, 2014; De Vries, 2004).
3. Chromosomal effects observed in intact mice could also be eliminated/reduced following gonadectomy. This would indicate that in the intact animal, hormones and chromosomes interact additively or synergistically to shape brain anatomy; we refer to this type of hormone-chromosome interaction as "cooperative effects" (Majdic and Tobet, 2011).

We observed compensatory and cooperative interactions between hormones and chromosomes in 18/64 structures in our mouse brain atlas, though they were of low magnitude. Within voxels where a main effect of chromosomes on volume was observed (such as the corpus callosum, septum, and dorsal striatum) the absolute percent difference between XY and XX mice was reduced by only 8% in gonadectomized animals, indicating that gonadectomy only subtly alters sex chromosome effects on brain anatomy. The fact that the average chromosomal effect is reduced when sex steroids are removed implies that overall there are more or larger cooperative effects than compensatory effects in the brain.

These findings also suggest that the interplay between sex chromosomes and sex hormones is more complex than previously thought.

There were 6 structures that displayed evidence of compensatory interactions between hormones and chromosomes. In the anterior commissure, ventral striatum, globus pallidus, internal capsule and BNST, the volume difference between XX and XY mice was enhanced after gonadectomy, indicating that circulating hormones can mask effects of sex chromosomes. One of the most intriguing findings was the emergence of chromosome effects in the BNST after gonadectomy. The BNST was smaller in gonadectomized XX mice versus XY mice, and this effect of chromosomes was two-fold larger in gonadectomized XX males than XX females. This indicates that hormones and sex chromosomes do interact in a complex manner to influence the structure of this critical sexually dimorphic structure.

We also examined the effects of early (in utero and pre-pubertal) versus late (pubertal and post-pubertal) hormones on neuroanatomy. Recent evidence suggests the classic model of hormonal wiring of the brain in two stages - organizational during perinatal development and activational in adulthood - is too simplistic (Arnold and Breedlove, 1985; McCarthy and Arnold, 2011). In fact, studies show that pubertal hormones can also have permanent differentiating effects, implying that the organizational window can extend to the end of adolescence (Ahmed et al., 2008; Juraska et al., 2013; Schulz et al., 2009).

Gonadectomy had a striking impact on the sex-hormone-dependent structural dimorphism observed in the intact mPOA. In intact mice, the mPOA is larger in males than females. This difference is driven by differences in sex hormones, not chromosomes (Corre et al., 2014; Gorski et al., 1978). Pre-pubertal gonadectomy reduced the mPOA volume in gonadal males to that of females but did not affect gonadal female volume. This indicates that pubertal and/or ambient male hormones are required

to establish or maintain the sex difference. This finding is consistent with studies demonstrating the role of androgens in masculinizing the mPOA. The sex difference in the mPOA is initially established via a perinatal surge in testicular hormones that promotes cell survival and increases dendritic spine density in males (Schwarz, 2016). Recent studies in rats indicate that pubertal hormones also shape mPOA volume by promoting cell proliferation, with more new cells being added in adolescent males than females (Ahmed et al., 2008). Pre-pubertal gonadectomy decreases cell proliferation in males only (Ahmed et al., 2008); thus the reduction in the mPOA volume observed in gonadectomized males relative to intact males may be due to reduced cell number. Additionally, changes in aromatase (which converts testosterone to estradiol) after gonadectomy may influence mPOA volume; the high aromatase levels in intact males is reduced to female levels following post-pubertal gonadectomy (Tabatadze et al., 2014). These data indicate that although the sex difference in mPOA volume originates early in development, it is either actively maintained and/or further programmed via pubertal or post-pubertal hormone effects. The mPOA modulates traits that appear after sexual maturation, such as copulatory and maternal behaviour (Dobolyi et al., 2014; Petrulis, 2013). It is interesting to speculate that areas of the brain that regulate post-pubertal behaviors may be especially sensitive to late hormonal effects.

The medial amygdala (MeA) is also larger in males (Hines et al., 1992). Intriguingly, we discovered that sex hormones and chromosomes exert opposing forces on MeA volume: the MeA is larger in 4CG gonadal males than females, but is smaller in mice with male chromosomes (XY) than those with female chromosomes (XX). Thus hormones and chromosomes appear to act in opposition to reduce what would otherwise be an even larger sex difference. Although androgens are known to increase MeA size, the molecular basis for the opposing sex chromosome effect is unknown (Morris et al., 2004). One possibility is that Y-specific genes (other than *Sry*) act to decrease MeA volume; alternatively, genes that escape X-inactivation and are expressed twice as much in XX vs XY

individuals (Raznahan et al., 2013) may act to increase MeA volume in XX mice. Although MeA volume was still larger in gonadectomized males versus females, the sex difference was reduced by approximately 66%, due to a significant volume reduction in males. Thus, like in the mPOA, the volume difference in the MeA is partly established via early hormone effects, but pubertal and/or post-pubertal male sex hormones are required to generate or maintain the full sex difference. This finding is consistent with molecular studies showing that both perinatal and pubertal hormones influence number and size of astrocytes and neurons in the MeA (Cooke et al., 2007; Dodson and Gorski, 1993; Pfau et al., 2016); accordingly, the reduction in MeA volume following pre-pubertal gonadectomy may be due to a reduction in neuron and/or astrocyte number. Interestingly, the chromosome-dependent volume difference between XX and XY mice was eliminated after gonadectomy, indicating sex chromosome effects on MeA volume require pubertal and/or post-pubertal sex hormone exposure to be manifested.

The BNST is another canonically sexually dimorphic region that is larger in males (Schwarz, 2016). This sex difference is established by perinatal gonadal steroids that promote cell survival/decrease cell-death in males, and it can be eliminated by neonatal gonadectomy of males or perinatal testosterone treatment of females (del Abril et al., 1987; Gotsiridze et al., 2007). Consistent with this, in intact 4CG mice, we found an effect of sex hormones but no effect of chromosomes on BNST volume, which likely arises from differences in cell number. In gonadectomized mice, although the BNST remained larger in males versus females, the sex difference was reduced by approximately 60%. Previous studies indicate that prepubertal gonadectomy leads to a loss of calbindin-positive neurons in gonadal males but does not affect cell number in gonadal females (Morishita et al., 2017). Thus the volume loss we observe is likely due to a reduction in this particular cell type. Again, these results suggests that pubertal and/or post-pubertal hormones are required for full sexual dimorphism in the BNST, as observed in human studies (Chung et al., 2002).

Interestingly, further analysis of the BNST data by genotype revealed that the attenuation of sex difference was mediated almost entirely by reduced volumes in XX males. These results indicate there is a surprising underlying effect of chromosomes on BNST volume that is normally masked by hormones.

There are several cellular mechanisms that could explain the complex effects of chromosomes and hormones on BNST volume. One hypothesis is that post-pubertal sex steroids are sufficient to maintain BNST volume, but in their absence, the expression of non-*Sry* genes on the Y-chromosome can compensate. Under this model, pre-pubertal gonadectomy would lead to a reduction in volume in gonadectomized XX but not XY mice. Alternatively, gonadectomy may alter the expression of X-linked genes, which could then decrease BNST volume in XX mice (Berletch et al., 2010). Indeed, several X-linked genes show sexually dimorphic gene expression patterns in the BNST that cannot be explained by escape from X-inactivation alone; moreover, gonadectomy has been shown to alter the expression of these genes in a sex-specific manner (Xu et al., 2012).

Overall, we found that hormonal-dependent sex differences in the volume of several canonically dimorphic brain regions were still present in gonadectomized mice; however, the extent of the difference was reduced (Figures 2 & 3). Within regions where sex hormones influenced volume, the percent difference between gonadal males and females was attenuated by approximately 58% in gonadectomized mice. These data imply that pre-pubertal hormone effects set up sex differences in the brain, but pubertal and/or post-pubertal hormones are required either to establish or maintain the full sex difference. Historically, perinatal hormonal effects have been assumed to be primary in generating sex differences; our results add to recent literature demonstrating an important role for pubertal and post-pubertal hormones in shaping brain anatomy.

Our whole brain analysis also revealed how hormones influence the anatomy of less well characterized brain regions. For instance, voxels within the superior colliculus and barrel/auditory cortices were significantly larger in females compared to males. Gonadectomy reduced these sex differences, indicating that both early and late hormone effects influence these regions as well. The barrel cortex displays interesting sex-specific responses, and some of those have been shown to be dependent on hormones, with female responses varying with the estrus cycle (Bobrov et al., 2014). A full histological characterization of the cellular basis of these effects is beyond the scope of this study; however, future molecular studies should probe the ontogeny of these newly identified and complex sex chromosome and hormone effects.

In addition to their effects on neuroanatomy, sex hormones and sex chromosomes also affect many behaviours. Previously, we found that gonadal males outperform gonadal females on a spatial RAM task, regardless of sex chromosome complement. Here we show that the male advantage persists even in pre-pubertally gonadectomized mice. This implies that pre-pubertal gonadal hormones establish spatial learning abilities in the RAM. These findings are consistent with both human and rodent studies demonstrating the permanent effects of early sex hormone exposure on spatial learning. For instance, men with congenital hypogonadism display impaired spatial cognition compared to males who develop hypogonadism post-pubertally (Hier and Crowley, 1982).

In summary, our findings expand our understanding of the roles of sex chromosomes and sex steroids in brain development, which is likely critical for understanding sex differences in the prevalence, age of onset, and symptomatology of neuropsychiatric diseases. Because male-biased disorders often have an early age of onset, they may be influenced by post-natal and pre-pubertal hormone levels as well as by the expression of X and Y linked genes. Intriguingly, in a recent study of multiple genetically-modified mouse models of autism, we found that affected brain regions clustered

into 3 distinct networks. One network included the BNST, amygdala, hypothalamus, and lateral septum (Ellegood et al., 2015). Here, we showed that many of these areas are indeed sensitive to early hormone effects, as well as to interactions between sex chromosomes and hormones. Meanwhile, neuropsychiatric disorders that are more prevalent in females, such as depression and anxiety, tend to arise during adolescence (Ahmed et al., 2008). In this regard, it is again intriguing that we identified several brain regions are sensitive to post-pubertal hormone effects, including the amygdala, a brain region commonly associated with emotional regulation. Studies investigating the origin and development of sex differences in the brain, and how sex hormones, chromosomes, epigenetic mechanisms, and social-environmental factors influence brain structure and function, will be critical to understanding the biological roots of the sexually dimorphic incidence rate in neuropsychiatric disease.

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