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Understanding Brain Sexual Dimorphisms and Reproductive Physiology in the Four Core Genotypes Rat Model

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Understanding Brain Sexual Dimorphisms and Reproductive Physiology in the Four Core Genotypes Rat Model

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by

Tara Rebecca Mohanroy

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ABSTRACT OF THE THESIS

Understanding Brain Sexual Dimorphisms and Reproductive Physiology

in the Four Core Genotypes Rat Model

by

Tara Rebecca Mohanroy

Master of Science in Physiological Science

University of California, Los Angeles 2022

Professor Arthur P. Arnold, Chair

Since the development of the Four Core Genotypes (FCG) mouse model, a variety of studies have identified sex chromosome effects on sex differences in behaviors (e.g., aggression and nociception), gene expression (e.g., septal vasopressin), and susceptibility to disease (e.g., multiple sclerosis and pulmonary hypertension). This is due to the ability of the FCG model to produce four unique groups (XX and XY gonadal males, XX and XY gonadal females) which allow for the independent comparison of gonadal hormone and sex chromosome effects that contribute to any phenotypic sex difference. A limitation of the model is that these effects can only be measured in mice, creating “mousified” results which might not reflect sex chromosome effects in other species. This has led to an attempt to create an FCG rat model which utilizes CRISPR technology to delete the testis-determining function from the Y chromosome, and transgenesis to insert an *Sry* transgene onto an autosome. Thus far, the model generates three types of gonadal males (XY wild-type, XY(*Sry*TG+), and XX(*Sry*TG+)) along with two types of gonadal females (XX and XY^{Δ}). The present study utilizes the FCG rat model to characterize sex differences in the cell number, cell size, and volume of the sexually

dimorphic nucleus of the preoptic area (SDN-POA) as well as the reproductive physiology between gonadal females (XX and XY^{Δ}). Brains from each of the five unique genotypes were collected and immunolabeled for calbindin (CALB) to identify the SDN-POA in the rat hypothalamus. The cell number, cell size, and volume of the nucleus were observed to be significantly larger in the gonadal males than in the gonadal females, suggesting the influence of gonadal hormone effects on the presence of the brain dimorphism. No differences were ascertained between XX and XY rats with the same type of gonad. No differences were noted in estrous cycle duration shows no significant differences between gonadal female groups (XX and XY^{Δ}) suggesting no sex chromosome effects. Taken together, these findings suggest that XX and XY rats with the same type of gonad appear to have comparable levels of gonadal hormones during development and adulthood, which cause sex differences in these dependent variables.

The thesis of Tara Rebecca Mohanroy is approved.

Stephanie M. Correa

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University of California, Los Angeles

2022

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INTRODUCTION

Sexual differentiation is defined as “the process by which the two sexes develop differences in any trait” (9). This process begins with inequalities present in development and continues on into adulthood, resulting in a multitude of sex differences between males and females. One of the most dominant presentations of these phenotypic differences is seen in the incidence and progression of various diseases. For example, women tend to suffer from autoimmune diseases more frequently than men, whereas men are more prone to brain deficits such as Parkinson’s disease or autism (26, 56, 57). While most diseases are treated similarly when presented in males and females, the presence of sex differences in disease requires an understanding of how sex modifies disease risk, progression, and treatment. These patterns imply the presence of sex-biased protective factors in one sex relative to the other which must originate from gonadal hormone effects or the direct actions of sex chromosome genes. If identified, the mechanisms influenced by these factors can provide novel entry points for more effective medical therapies.

For the past century, the cause of this sexual differentiation process was thought to be solely attributed to the influence of gonadal hormone levels at various sensitive periods in life. Female gonads secrete predominantly estrogen and progesterone while male gonads secrete predominantly testosterone, which serve as causal factors in the development of specific traits and behaviors. These findings were first reported by pioneers such as Lillie et al., who concluded that males have masculinizing factors after observing abnormal development of the internal and external genitalia in an intersex calf caused by atypical levels of prenatal hormones (37). This was later expanded upon by Jost et al., who conducted a series of experiments that led him to conclude testosterone and anti-Mullerian hormone were the masculinizing factors secreted by the testes which controlled the organization of male development. Jost further stated that female development was the “default” pathway that occurred when neither of these hormones were present (34). Finally, Phoenix et al. identified organizational (“permanent”) and

activational (“reversible”) effects of gonadal hormones by exposing neonatal female guinea pigs to testosterone and observing masculinized reproductive behaviors in adulthood (4). Together, these ideas led to the widespread acceptance of the hormonal theory of sexual differentiation, otherwise known as the “classic theory”, which has heavily influenced many experiments and scientific discoveries regarding sex differences (Figure 1) (40).

While most sex differences were initially reported on reproductive tissues, studies on sex differences in behavior led to a shift towards understanding non-reproductive phenotypes and structural dimorphisms in neural circuitry. This interest originated from observed behavioral differences between males and females seen in sexual behavior and maternal behavior (36). The identification of these brain sexual dimorphisms provides insight into the cellular and molecular biological factors caused by the organizational and activational effects of gonadal hormones. Sex steroids act to influence the outgrowth of axons and dendrites, onset of cell death, and the number of synaptic connections made in each region of interest (24). Studies originally performed using rats as a model organism, identified dimorphic regions in the central nervous system such as the spinal nucleus of the bulbocavernosus (SNB), arginine vasopressin (AVP) innervation of the lateral septum, the anteroventral periventricular nucleus (AVPV), the bed nucleus of the stria terminalis (BNST), and the central part of the medical preoptic nucleus (MPNc) (11, 22, 42, 51, 31, 35). When measuring variables such as cell density, cell number, dendritic branching, and volume, all of the regions were found to be primarily male dominant (males > females), with the AVPV being the only cell group that was female dominant (females > males).

The goals of this study specifically focus on understanding the mechanisms that control sex differences in the central part of the medical preoptic nucleus (MPNc). The MPNc was originally discovered in 1978 and named the sexually dimorphic nucleus of the preoptic area (SDN-POA) by Roger Gorski and colleagues at the University of California, Los Angeles. Using a Nissl stain, he identified the volume of the SDN-POA to be approximately 5 times greater in

male rats compared to female rats, which provided further evidence for the sexual differentiation of brain function (28). This brain dimorphism was also found to be conserved across multiple organisms such as mice, gerbils, guinea pigs, and humans (21, 30, 31, 33). The medial preoptic area is involved in the regulation of sexual behavior, however the specific function of the SDN-POA remains unknown. Bilateral lesions made in the SDN-POA in adult male rats resulted in increased latency to the first mount, intromission, and ejaculation, with other studies also finding an effect on partner preference (3, 16, 21). However, some negative results have also been reported with lesions in specific locations of the SDN-POA resulting in no changes in male reproductive behavior (3).

When male rats were castrated neonatally, the volume of the adult SDN-POA was reduced significantly compared to male rats that were castrated during the weaning period which suggested a time dependent hormonal influence on the dimorphic organization of the cell group. The aromatization of testosterone to estradiol, was found to be necessary for the masculinization of the SDN-POA (31). Rhees et al. later confirmed that the sensitive period, which resulted in the organizational effects of androgens on the development of the SDN-POA, occurred through postnatal day 5 after which the nucleus became insensitive to any further exposure to testosterone (47). Many sex differences in the brain are caused by hormonal regulation of cell death. However, the cell death regulator *Bax* gene, which has been found to be required for hormonal induction of other prominent brain dimorphisms, does not appear to be required for sexual differentiation of the SDN-POA (25). Recent studies have suggested glial cells as a mediator of cell death in the preoptic area as seen with changes in dendritic spine density, however a confirmed physiological mechanism has yet to be identified (41, 53).

Although initial studies on the SDN-POA were reported using Nissl stains, calbindin (CALB) has since emerged as a specific marker of the cell group (50). Calbindin-D28k is a calcium-binding protein which is known to play a role in neuroprotection by acting as a cytosolic calcium

buffer. In the current study, CALB immunoreactive (CALB-ir) cells were used to define the SDN-POA.

Although there is no doubt concerning the role of gonadal hormones on sexual differentiation, sex chromosome genes also cause sexual differentiation. The study of a rare bilateral gynandromorphic zebra finch suggested a role for non-gonadal factors. The finch had genetically male tissue (ZZ) predominating on one side of the body, and genetically female tissue on the other side (ZW) (1). The male side had a testis, and the female side an ovary. The high vocal center (HVC) which controls the neural circuit for song, and is known to be much larger in typical males than females, was larger on the genetically male side of the brain relative to female side. Because gonadal hormones would have influenced both sides of the brain equally, the lateral difference in sexual phenotype was associated with the complement of sex chromosomes rather than the level of gonadal hormones. The results of this study suggest that the sexual phenotype of the brain is controlled by both genetic sex and hormonal environments.

Burgoyne et al. contributed to the sex chromosome theory with the discovery of differences in the development and size of XY and XX mouse embryos prenatally prior to gonadal sexual differentiation. They concluded that the reason XY embryos were developmentally more advanced and larger than XX embryos was due to a combination of Y chromosome effects and the effect of X chromosome complement. This effect was also found to be present both before and after implantation of the embryo, well before the formation of gonads (12).

During development, the sex chromosomes themselves differ between males and females in multiple ways: (1) Males are the only sex that have Y genes creating male-specific effects, (2) X chromosome complement varies between sexes with X-inactivation only occurring in females and leading to higher expression of X escapee genes, and (3) females tissues are mosaics of cells with either a maternal or paternal imprint which contribute to differences in tissue function (5). Investigators began to consider X and Y genes to be primary causal factors

that stimulated sex differences in development in a more parallel-interactive model of sexual differentiation that encompassed the interactions of various biological factors and the environment on complex molecular pathways (Figure 1) (40). While the hormonal theory focused solely on the role of gonadal hormones on non-gonadal tissues creating sex-specific phenotypes, this “unified” model establishes the role of hormonal factors, genetic factors, and other secondary effects on the sexual differentiation of all tissues and other phenotypes (4).

Studying the independent effects of sex chromosomes poses a challenge because of the difficulty of altering sex chromosomes without simultaneously changing gonadal hormone levels. This led to the development of the Four Core Genotypes (FCG) mouse model (Figure 2), pioneered by Paul Burgoyne and Robin Lovell-Badge, which allows for the independent comparison of sex chromosome complement (XX vs. XY) while keeping the gonads the same (in mice with testes or ovaries) (4). Deletion of the testis-determining gene, *Sry,* from the Y chromosome (Y^-) resulted in the development of an XY gonadal female. This allows for the comparison of XY^- and XX gonadal females. To make the XX vs. XY comparison in gonadal males, an *Sry* transgene was inserted onto chromosome 3 leading to the development of testes, allowing for the comparison of XY^- (*Sry*+) and XX (*Sry*+) gonadal males (23). With this model, the effects of sex chromosomes can be measured independently of gonadal hormones (i.e., XX gonadal male vs. XY gonadal male) and the effects of gonadal hormones can be measured independently of sex chromosomes (i.e., XX gonadal female vs. XX gonadal male). Thus far, multiple studies have used the FCG mouse model to identify numerous sex chromosomal effects in various diseases while eliminating the confounding effects of gonadal secretions. These findings include sex chromosome effects in multiple sclerosis (XX > XY), Alzheimer’s disease (XY > XX), pulmonary hypertension (XX > XY), and adiposity (XX > XY) (14, 20, 55, 56). Further analysis revealed these sex differences to be caused by the expression of sex chromosome genes such as: (1) *Kdm6a*, an X-escapee gene which creates sex differences in

multiple sclerosis and Alzheimer's disease (32). (2) *Uty,* a gene found on the Y chromosome which serves a protective role in pulmonary hypertension (18), and (3) *Kdm5c*, an X-escapee gene found to contribute to differences in body weight and adiposity (38). In addition, studies using the FCG mouse model confirmed the influence of gonadal hormones on sexual brain dimorphisms previously established in brain regions controlling sex-specific social and reproductive behavior in rats (23). The results of these experiments also uncovered a sex chromosome effect on AVP innervation of the lateral septum, demonstrating the effectiveness of the FCG model and uncovering other mechanistic pathways that might contribute to the presence of this sex difference.

The FCG model was originally produced and studied using mice as a model organism. The size and ease in creating transgenic models led to mice being predominantly used to study mechanistic biomedical questions. Many novel discoveries in disease mechanisms and brain dimorphisms have resulted from the use of mouse models. Nevertheless, it is critical to investigate any important biological question in more than one species, to achieve a general answer. Accordingly, a rat FCG model was created (Figure 3). The rat model now allows for the comparison of XX and XY rats with the same type of gonad, to detect sex chromosome effects causing sex differences in rats. Indeed, rats are superior models of some aspects of human physiology and disease (43). Thus, it is expected that the FCG rat model will uncover new sex chromosome effects that could not be discovered in mice.

While the FCG model in the mouse required the deletion of a single *Sry* gene, the rat model has 11 *Sry* genes. The presence of multiple *Sry* genes creates a challenge in identifying which genes cause the development of the testes. Using RNASeq analysis, *Sry*4a was found to be the most expressed *Sry* gene along with *Sry1* and *Sry3c* at E13, the time of testis differentiation during embryonic development (46). Using a bacterial artificial chromosome (BAC) clone, a single BAC was inserted into rat embryos which contained a ~160KB genomic sequence encoding all three of these relevant *Sry* genes, but no other *Sry* genes. This allowed

for the creation of FCG founder lines which were able to produce XX gonadal male offspring. This result proves that 3 *Sry* genes (*Sry4a, Sry1, Sry3c)* were sufficient to cause differentiation of testes. To produce the *Sry* knockout rats, CRISPR gRNAs were used to target flanking sequences around *Sry4a*, deleting *Sry* regions and producing XY^{Δ} gonadal females that can reproduce. Although the deleted portion of the Y^{Δ} chromosome has not been defined, it is likely to include *Sry4a, Sry1, Sry3c, and Rbmy.* The Y^{Δ} still retains other Sry genes, which are not adequate by themselves to cause differentiation of testes. The FCG rat model is made using two crosses in contrast to the FCG mouse model which is made with one cross. The “female half” of the rat model was created by breeding XY^{Δ} gonadal females with XY WT males to produce XX and XY^{Δ} gonadal females for comparison. The “male half” of the rat model involves breeding XY(*Sry*TG+) males with XX WT females to produce 3 types of males for comparison, XY WT, XX(*Sry*TG+*),* and XY(*Sry*TG+).

To confirm that the hormone levels of the gonadal male and gonadal female FCG rat groups were comparable during development, anogenital distance, a trait correlated with gonadal steroid levels, was measured during the first postnatal week. In adulthood, serum levels of testosterone were measured (15). The results of both bioassays showed significant differences between the gonadal male and gonadal female groups, but no differences within groups containing the same gonads. These findings imply that the effects of gonadal hormones are comparable among rats with the same gonads. This conclusion suggests that the comparison of XX and XY rats with the same gonads is not confounded by major group differences in levels of gonadal hormones.

The primary goal of this project is to use the FCG rat model to study and quantify major sexual brain dimorphisms, specifically in the SDN-POA. Additionally, we measured the duration of estrous cycles between XX and XY^{Δ} gonadal female rats to compare reproductive physiology and produce timed pregnancies. Although sex differences in the SDN-POA were originally discovered in rats and later identified using the FCG mouse model, replicating the experiments

using the FCG rat model will provide several benefits. First, if the results are found to replicate has been reported for the FCG mouse model, this will serve as evidence for the conservation of the brain dimorphisms across multiple model organisms. This is important, as model organisms are used to perform experimental manipulations with the goal of uncovering developmental mechanisms that are potential targets against human diseases. It is assumed that when these mechanisms are consistent across multiple model animals, they provide greater generalizability about the understanding of these mechanisms in humans. Next, completing this study will allow for the effects of sex chromosomes and gonadal hormones on these brain dimorphisms to be independently tested for in the FCG rat model. Applying these findings to the FCG mouse model has resulted in convincing evidence that these brain dimorphisms are heavily driven by gonadal hormones**.** As these studies have primarily been done in mice, expanding these results using the FCG rat model will allow for the main effect of gonadal hormones to be further tested and confirmed. As the rat and mouse genome are not completely identical, this could also uncover potential sex chromosome effects that were not seen in the FCG mouse model. Finally, using the FCG rat model for better understanding of these sex differences provides confirmation of the utility of the FCG model for understanding complex tissue and disease systems. If these regions are found to be predominantly masculine in the gonadal male FCG rats (XX and XY rats with testes) and feminine in the gonadal female FCG rats (XX and XY rats with ovaries), this will be convincing evidence that this comparison is possible while holding hormones as constant as possible. This is the strength of the FCG model compared to other models used for similar purposes, as each potential causal factor (sex chromosomes, gonadal hormones) can be independently manipulated while replicating differences in normal males and females. Ultimately, this study will allow us to advance our understanding of sexual differentiation in the brain and provide knowledge on sex differences in behavior and disease.

MATERIALS AND METHODS

Animal Care

Founder *Sry*-knockout transgenic Sprague Dawley rats (strain SAS CD 400) were created at the Medical College of Wisconsin by our collaborators. Daughters of the founder XY^{Δ} females were shipped to UCLA and backcrossed to Sprague Dawley CD (001) for 4-6 generations prior to experimentation. Founder *Sry*-transgenic rats (Sprague Dawley CD (001)) were produced at the University of Michigan Transgenic Animal Core, and shipped to UCLA where they were then backcrossed to Sprague Dawley CD (001) for 4-8 generations prior to experimentation. Breeding and experimentation were done at the University of California, Los Angeles. The rats were maintained on a 12:12 light-dark cycle and procedures were conducted in accordance with protocols approved by the UCLA Chancellor's Animal Research Committee (ARC).

The Four Core Genotypes rat model consists of five unique genotypes that allow for the independent comparison of gonadal hormone and sex chromosome effects. The "male-half" of the model was created through breeding an XX gonadal female with an XY (*Sry*TG+) to produce XY WT, XX (*Sry*TG+), and XY (*Sry*TG+) gonadal males. The "female-half" of the model was created through breeding an XY^{Δ} gonadal female with an XY WT gonadal male to produce XX wild-type and XY^{Δ} gonadal females.

Estrous Cycle Analysis

Rats from XY^{Δ} mothers were genotyped with PCR prior to any experimentation to measure the presence of the Y chromosome and discriminate XX vs. XY^{Δ} gonadal females. After reaching approximately 8 weeks of age, vaginal smears were collected daily between 12:00pm and 1:00pm using an established vaginal swabbing technique to quantify an estrous cycling pattern (2, 17). Each collected smear was staged the same day, and the cytological

patterns of cell type and cell shape were considered prior to confirming the stage of estrous (Figure 4). Staging was done for about 3-4 consistent cycles before timed pregnancies were attempted, in which the females were identified to be in estrus and placed with a male rat overnight. The length of each cycle prior to the timed pregnancy was recorded for all female rats used for this experiment, and the average length of each animal was reported and compared across groups.

Immunohistochemistry

Rats were anesthetized with isoflurane and decapitated. Brains were removed, fixed with 4% paraformaldehyde (PFA), and cryoprotected with 30% sucrose and frozen at -80°C for subsequent processing with immunohistochemistry. Consecutive 40μm coronal cryostat brain sections were collected and briefly placed in a 0.1M glycine solution for 30 minutes. Sections were then washed and incubated at room temperature in a blocking solution made with 3% hydrogen peroxide, 10% normal horse serum (NHS), 0.4% Triton X-100, and tris buffered saline (TBS) for an additional 30 minutes. Then, sections were incubated in a primary antibody cocktail solution directed against CALB (Mouse Monoclonal Anti-Calbindin-D-28K (Cat #C9848, Sigma, St. Louis, MO)) with a 1:10,000 dilution in 2% normal horse serum, 0.4% Triton X-100, and TBS for 48-72 hours at room temperature. All sections were then washed in TBS and placed in a secondary antibody cocktail solution made with biotinylated horse anti-mouse immunoglobulin G (IgG) secondary antibody (1:500, Vector Labs, Burlingame, CA) for 60 minutes at room temperature. The signal was amplified using an avidin-biotin complex kit (Vector Labs, Burlingame, CA) and enhanced using a DAB chromagen (Sigma, St. Louis, MO). After completing a final round of washes with TBS, the sections were mounted sequentially onto Fisherbrand Superfrost Plus slides (Fisher, Pittsburgh, PA) and allowed to dry overnight in a dark space. The sections were then dehydrated in a series of washes with increasing ethanol

concentrations and cleared in Hemo-de (Fisher, Pittsburgh, PA) before being coverslipped with DPX mountant (Sigma, St. Louis, MO).

Quantification of calbindin (CALB)-immunoreactivity in the SDN-POA

Brightfield microscopy was used to identify the SDN-POA at low magnification (5x) and further measurements for cell number, cell size, and nucleus volume were acquired at high magnification (63x) with oil immersion. The anterior commissure was used as a marker to identify the beginning of the SDN-POA. The presence of CALB-immunoreactive (ir) cells located more caudally in the BNST served as a marker for the end of the SDN-POA nucleus (53). CALB-ir cells within the SDN-POA region were found within 5-6 sections in males and 2-3 sections in females. CALB-ir cell number was estimated by visual inspection using the optical dissector method and collected manually using CALB-ir cells found within the nucleus. The total cell number was reported as a sum of the counts collected from both hemispheres across sections containing the SDN-POA. Images of the nucleus found on the left and right hemisphere were processed in ImageJ in order to quantify cell size and nucleus volume. A calibration measurement was taken to ensure all values were reported in units of millimeters. The areas of five randomly selected cells were taken from each hemisphere on each section, resulting in approximately 50-60 cell areas collected from the male SDN-POA and 20-30 cell areas measured from the female SDN-POA. The mean cell size was calculated for each animal from all the cells that were measured. The volume of the CALB-ir region of the SDN-POA was also quantified with ImageJ, using the segmented line tool to define the outer border of each sampled cell calculate the area of each section containing the nucleus and then multiplying each value by the section thickness (40μm). The total reported volume was a sum of these individual section volumes. Images were captured using a Nikon D750 DLSR camera mounted on a Leica DMRXA scope (Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

Experimental groups were compared using a one-way analysis of variance (ANOVA) with a post-hoc Tukey-Kramer test to conduct pairwise comparisons. All analysis was completed using the NCSS statistical software.

RESULTS

Comparison of Estrous Cycle Duration

Samples of vaginal smears were collected and the state of estrus was characterized based on cell populations and clustering patterns. During proestrus and estrus, epithelial cells made up the majority of the cell population in each sample. Specifically, nucleated epithelial cells were found predominantly in proestrus and cornified epithelial cells were found clustered together in estrus. During metestrus, the samples consisted of a mix of epithelial cells and neutrophils, with diestrus containing mostly all neutrophils which were scattered across the sample. These cytological patterns are illustrated in Figure 4 and were found to be consistent with previously reported findings. This served as a reliable metric for the identification of each estrous cycle stage (17).

After collecting data on estrous cycle duration for 3-4 complete cycles, estrous cycle duration was found to last approximately 4-5 days for both groups of gonadal females, with the length being 4.69 ± 0.17 days for XX WT and 4.31 ± 0.16 days for XY^{Δ}. A one-way ANOVA revealed there to be no significant differences between the average estrous cycle duration of XX and XY^{Δ} gonadal females, further suggesting no sex chromosome effects on reproductive physiology ($p > 0.05$).

Cell Counts

The number of CALB-ir neurons in the SDN-POA was found to be sexually dimorphic with gonadal males having more than twice the number of CALB-ir neurons than gonadal females. This finding replicated the sex difference previously reported in studies of rats and mice. The average cell count for XY WT was 1006 ± 42 with the average cell count for XX WT being 441 ± 28. The summarized results of cell number in the SDN-POA for all genotypes within

the FCG rat model can be found in Table 1. The one-way ANOVA revealed a significant difference in average cell counts among the genotypes compared ($p < 0.001$). The results of the pairwise comparisons made with using a post-hoc Tukey-Kramer test identified significant differences between the gonadal female groups and the gonadal male groups ($p < 0.05$), with the mean cell count values being larger in the males than the females. This confirms that the presence of sex differences in SDN-POA CALB-ir cell number are hormone-dependent, with no significant effect of sex chromosomes. Within groups, the gonadal male genotypes did not differ significantly from each other and the gonadal female genotypes also did not differ significantly from each other ($p > 0.05$).

Cell Size

Past studies have identified sex differences in the size of neurons within the medial preoptic area (39). This led to the investigation of cell size differences in the SDN-POA using the FCG rat model to identify sex-biased causal factors. After calculating the mean cell size from approximately 50-60 cells from each male SDN-POA and 20-30 collected cells from each female SDN-POA, CALB-ir cell size was found to be about 1.7 times larger in gonadal males than gonadal females. This can be seen with the average cell size of XY WT was 3.33 ± 0.08 ($mm^2 \times 10^{-5}$) and 1.90 ± 0.07 ($mm^2 \times 10^{-5}$) for XX WT. The summarized results of quantified cell sizes in the SDN-POA using the FCG rat model can also be found in Table 1. The one-way ANOVA indicated that the five groups showed significant differences in cell size ($p < 0.001$). Post hoc Tukey-Kramer tests indicated that there was a significant difference identified between gonadal male groups and gonadal female groups, confirming a main effect of gonadal hormones causing sex differences in cell size within the SDN-POA ($p < 0.001$). Additionally, there were no significant effects of sex chromosomes on cell size, and no differences identified between groups of the same sex ($p > 0.05$).

Nucleus Volume of the SDN-POA

Initial studies which led to the discovery of the SDN-POA and its dimorphic nature, quantified sex differences in nucleus volume which correlated with differences in reproductive behavior. In the present study, the female SDN-POA CALB-ir was noticeably smaller in size with the neurons more densely packed together. The nucleus itself was contained within 2-3 coronal sections for about 80-120μm in the rostro-caudal axis. In comparison, the male SDN-POA CALB-ir was much larger in size with the nucleus present within 5-6 coronal sections with neurons dispersed across approximately 200-240μm in the rostro-caudal axis. On average, the volume of the SDN-POA CALB-ir was found to be about 3 times greater in males than in females, with a volume of 3.02 ± 0.17 ($mm^3 \times 10^{-3}$) for XY WT males and 1.07 ± 0.12 ($mm^3 \times 10^{-3}$) for XX WT females. The reported values of SDN-POA CALB-ir volumes for all genotypes within the FCG rat model can be found in Table 1. One-way ANOVA revealed a significant difference among groups ($p < 0.001$), with the Tukey-Kramer tests showing significant differences between gonadal female groups and each gonadal male groups ($p < 0.05$). The pairwise comparisons also confirmed no effect of sex chromosomes on the presence of sex differences in the volume of the SDN-POA. There were also no significant differences of SDN-POA CALB-ir nucleus volume within groups of the same sex ($p > 0.05$)

DISCUSSION

The aim of the present study was to measure estrous cyclicity and the SDN-POA, a major morphological sex difference in the brain of rats, in the new Four Core Genotypes rat model. We sought to determine, for the first time, if sex chromosome complement (XX vs. XY) contributes to sex differences in structure of the SDN-POA, or to differences in estrous cycle length. We found no effects of sex chromosome complement. We also wished to determine if groups with the same type of gonad, but with different sex chromosomes, have comparable levels of gonadal hormones, using the dependent variables as indicators of the influence of gonadal hormones during ontogeny. We found no differences in dependent variables among rats with the same gonads, suggesting that they had comparable levels of gonadal hormones during critical periods of sexual differentiation of these parameters.

Estrous Cycling

Estrous cyclicity was found to be similar between XX and XY^{Δ} gonadal females which confirms the absence of sex chromosome effects on estrous cycle length. Estrous cycles are characterized by morphological and physiological changes in the ovaries, uterus, and vagina, all of which occur in response to circulating estrogens and progestins. The length of the estrous cycle requires appropriate functioning of a complex circuit of neurons and astrocytes in the hypothalamus, that regulate the release of anterior pituitary gonadotropins and induce the maturation, ovulation, and formation of the corpus luteum in the ovary. The interactions of these tissues are fine-tuned and regulated by diverse hormonal signals such as gonadotropin releasing hormone, follicle-stimulating hormone, luteinizing hormone, estradiol, and progesterone. The similarity in estrous cycling found in XY^{Δ} and XX females in the present study suggests that sex chromosome complement did not cause major differences in this complex neuroendocrine axis. For example, the presence of the Y^{Δ} does not interfere with ovarian cycling, even though this chromosome has some Sry genes remaining, which are not sufficient

to act as the testis-determining factor. The results of this study confirm there to be no additional sex chromosomal effects on estrous cycling when making comparisons with gonadal females (XX and XY^{Δ}). If found, the presence of sex chromosome effects would have served as a confounding variable when making comparisons between the gonadal females. The absence of sex chromosome effects on estrous cycling provides further evidence that the levels of gonadal hormones are comparable between the gonadal female groups.

In mice, XY gonadal females are often subfertile, depending on which strain is used (13). The strains of mice showing greatest fertility of XY females are outbred mice. In FCG rats, the background strain is Sprague Dawley, also an outbred strain, which may explain why possession of a single X chromosome, or the Y^{Δ} chromosome, do not interfere with estrous cycling in FCG rats.

The Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA)

The SDN-POA was found to have larger CALB-ir cell number, cell size, and overall nucleus volume in all gonadal male groups (XY WT, XY(*Sry* TG+), XX(*Sry* TG+)) compared to gonadal female groups (XX WT, XY^{Δ}). Because the SDN-POA is considered a classic brain dimorphism and also found to be conserved in a variety of model organisms (i.e., mice, rats, ferrets, gerbils, etc.), we hypothesized that the sex difference would be replicated with the FCG rat model. Previous studies have identified the most 'sensitive period' of sexual differentiation of the SDN-POA to be between embryonic day 18 and postnatal day 4, with the administration of testosterone or oestradiol to female rats during this period causing masculinization of the SDN-POA volume (49). Females lose a large number of neurons between postnatal day 4 and postnatal day 10, compared with males that show no significant decrease over this time period (20). The FCG rat model revealed the number of CALB-ir cells within the SDN-POA to be affected by the type of gonadal hormones, and with gonadal males having a larger cell number than gonadal females, confirms the long-standing conclusion that sexual differentiation of the

SDN-POA is caused by gonadal hormones. Previous studies suggest the sex difference in cell number is likely a result of sex steroid-regulated cell death (27). Our results do not provide any evidence that sex chromosome complement contributes to sexual differentiation of the SDN-POA in rats, a novel finding of the current study. Previous studies of FCG mice also fail to show any sex chromosome effect on hypothalamic sexual differentiation (22), although that study measured a different hypothalamic nucleus, the AVPV (anteroventral periventricular nucleus).

One goal of studying the SDN-POA was to utilize the nucleus as an established measure of the organizational effects of gonadal hormone levels across the five genotypes within the FCG rat model. We asked if rats with the same type of gonads, but with different sex chromosomes, have similar metrics of brain structure which are known to reflect the action of gonadal hormones at the developmental stages when these hormones regulate SDN-POA development. The similar morphology (cell number, cell size, and overall nucleus volume) of the SDN-POA within gonadal males, provided evidence that the testes in the XX gonadal males secreted enough perinatal testosterone to masculinize the nucleus to the same degree as the XY wild-type males. In females, the similarity of SDN-POA morphology in two gonadal female groups suggests that XY^{Δ} female do not secrete significant amounts of androgens. These results provide evidence that the gonadal hormone levels of all male groups are comparable, and are unlikely to explain any group differences found when comparing XX and XY gonadal females.

Although it is suspected that the presence of the sexual dimorphism in cell number in the SDN-POA is influenced by hormone-mediated cell apoptosis, the mechanism by which cell death is initiated remains unknown. Calbindin was used as a marker for a defined population of cells found within the nucleus. Interestingly, calbindin, a calcium-binding protein, is known to have a role in preventing neuronal cell death through its role as an intracellular calcium buffer (48). Calbindin appears to protect neurons from harmful increases in intracellular calcium which may initiate cell-death mechanisms. With the results of this study revealing FCG gonadal males

to have greater SDN-POA CALB-ir cell number, cell size, and nucleus volume compared to gonadal females, a future study could conduct a series of hormone manipulations to quantify the influence of gonadal hormones on the mechanisms which calbindin acts upon to promote apoptosis. Additionally, anterograde tracing techniques could be used to identify termination points of axonal projections from hypothalamic neurons in the SDN-POA to better understand the neuronal pathways which initiate male reproductive behavior controlled by the nucleus. Identifying these targets could help uncover the direct role of the SDN-POA in the hypothalamic circuit of male reproduction, and explain the reasoning behind its highly conserved dimorphic nature across multiple animal models.

Currently, our lab is also studying another neural sexual dimorphism, in the AVPV, using the FCG rat model. This nucleus is slightly different from the other nuclei mentioned, as the AVPV is found to be larger in females compared to males (50). Similar to the SDN-POA, the AVPV is rostral to the MPNc and past studies have identified the dimorphic organization of the nucleus to be dependent on perinatal levels of gonadal hormones (31). If the cell number and average cell size of the AVPV is found to be larger in FCG gonadal females compared to FCG gonadal males, this will provide additional evidence for the model in its ability to create XX and XY animals with the same gonad that have comparable levels of hormones.

CONCLUSION

In summary, we sought to understand reproductive physiology and neural sexual dimorphisms using the Four Core Genotypes rat model. The ultimate goal of this model was to expand on findings discovered using the Four Core Genotypes mouse model to study physiological mechanisms in multiple model organisms and achieve more generalizable results We used estrous cycle length as a measure of reproductive fit and found estrous cycling to be similar between XX and XY^{Δ} gonadal females. Additionally, these findings concluded that there were no sex chromosome effects on estrous cycle length. Using CALB-ir neurons as a marker for the SDN-POA, I observed significant differences identified between all gonadal male and gonadal female groups in cell number, cell size, and overall nuclear volume. Gonadal male groups were found to have a larger SDN-POA compared to gonadal female groups which confirmed the results of past findings. The results of this study also identified the dimorphic organization of the SDN-POA to be hormone-dependent with no influence of sex chromosome effects, suggesting that XX and XY animals with the same gonads have comparable levels of gonadal hormones. These findings contributed to providing compelling evidence for the effectiveness of the FCG rat model and its ability to make 2 x 2 comparisons between groups while measuring gonadal hormone effects and sex chromosome effects independently of each other.

FIGURES

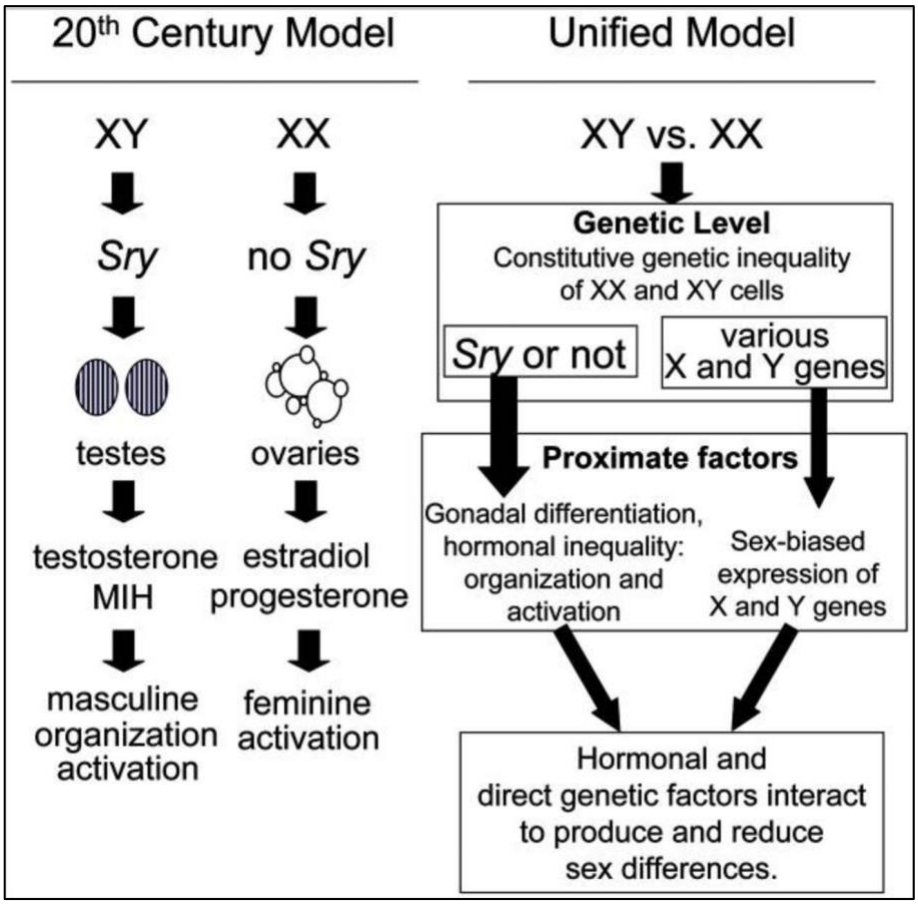

Figure 1: The 20th Century Dogma of Sexual Differentiation vs. the 21st Century Dogma of Sexual Differentiation. The 20th Century Dogma of Sexual Differentiation, also known as the hormonal theory, indicates that gonadal hormones are the main proximate factors causing sexual differentiation of tissues. The 21st Century Dogma of Sexual Differentiation, also known as the parallel-interactive model, suggests a unified contribution of gonadal hormones and sex chromosomes to sexually differentiate various tissues.

Note: Reprinted from "The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues," by Arthur P. Arnold (2009). *Hormones and behavior,* 55(5), 570-578.

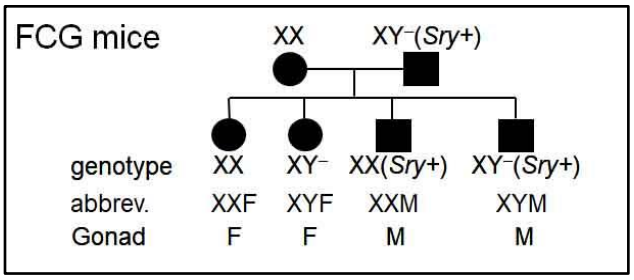

Figure 2: Four Core Genotypes (FCG) Mouse Model Breeding Scheme. The "female half" of the FCG mouse model is represented by the XX and XY- genotypes and the "male half" of the FCG mouse model is represented by the XX(*Sry*+) and XY-(*Sry*+) genotypes. This model allows for a 2 x 2 comparison to be made with gonadal hormones or sex chromosome complement as the independent variable.

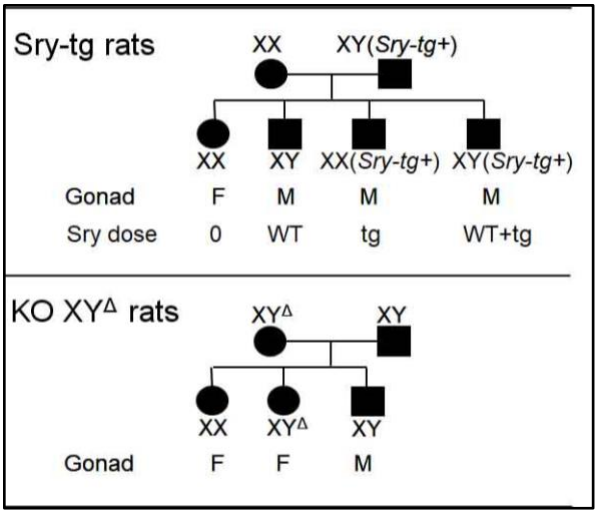

Figure 3: Four Core Genotypes Rat Model Breeding Scheme. The gonadal female FCG rats were produced by breeding XY$^{\Delta}$ female rats with an XY wild-type male rats. The gonadal male FCG rats were produced by breeding XY$^{\Delta}$ female rats with XX (*Sry* TG+) male rats. Similar to the FCG mouse model, the FCG rat model allows for the comparison of different phenotypes and traits to be made with gonadal hormones and sex chromosome complement as the independent variable.

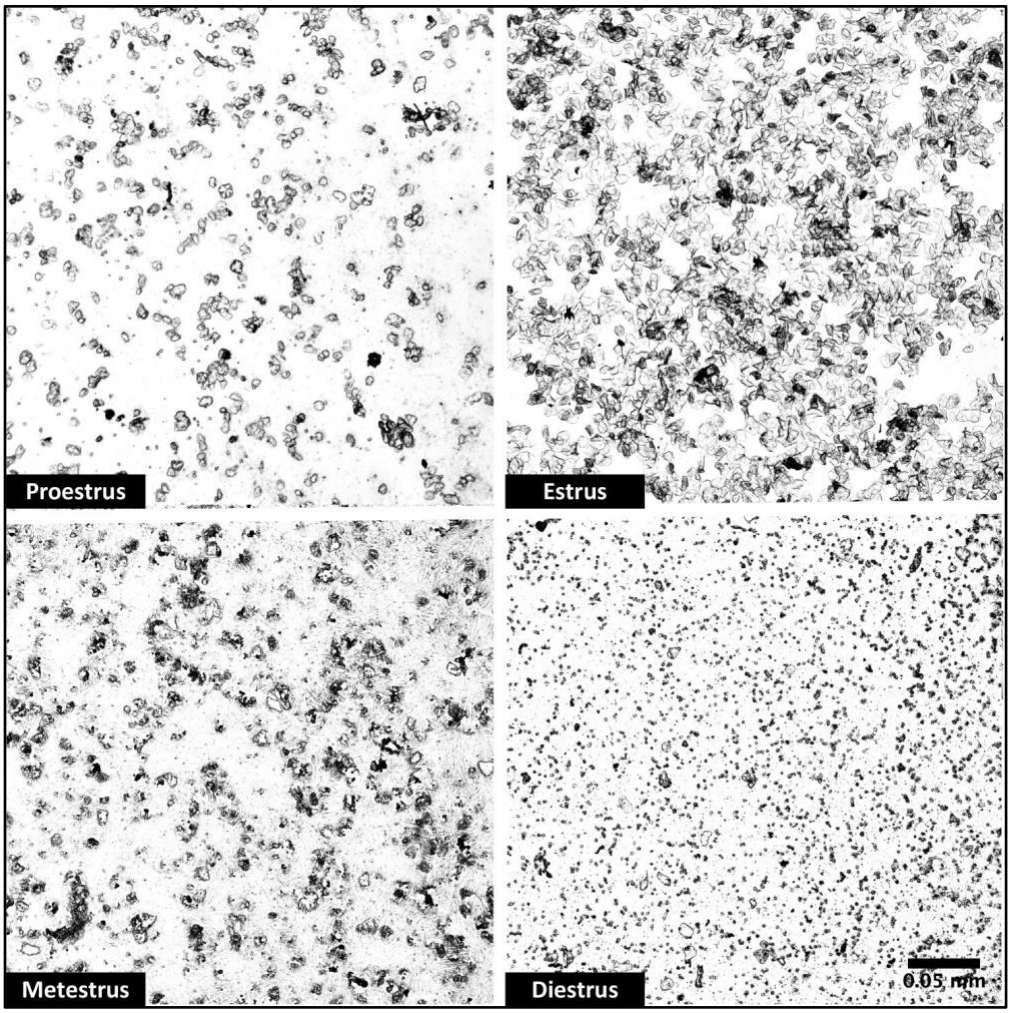

Figure 4: Vaginal Cytology of Estrous Cycle Stages in Wild-Type Female. Photomicrographs were taken at 5x magnification (scale bar: 0.05 mm) and show typical epithelial cell and neutrophil cell populations over a full estrous cycle (4-5 days). The cytological patterns of thee cell types were used to identify each estrous stage. Proestrus was defined by the presence of rounded and nucleated epithelial cells seen in small, cohesive clusters. Estrus was identified with high populations of cornified epithelial cells organized in large stacks or layers. Metestrus was defined by a mix of anucleated, keratinized epithelial cells and neutrophils. And diestrus was determined by identifying the large cell population of neutrophils with sparsely scattered epithelial cells.

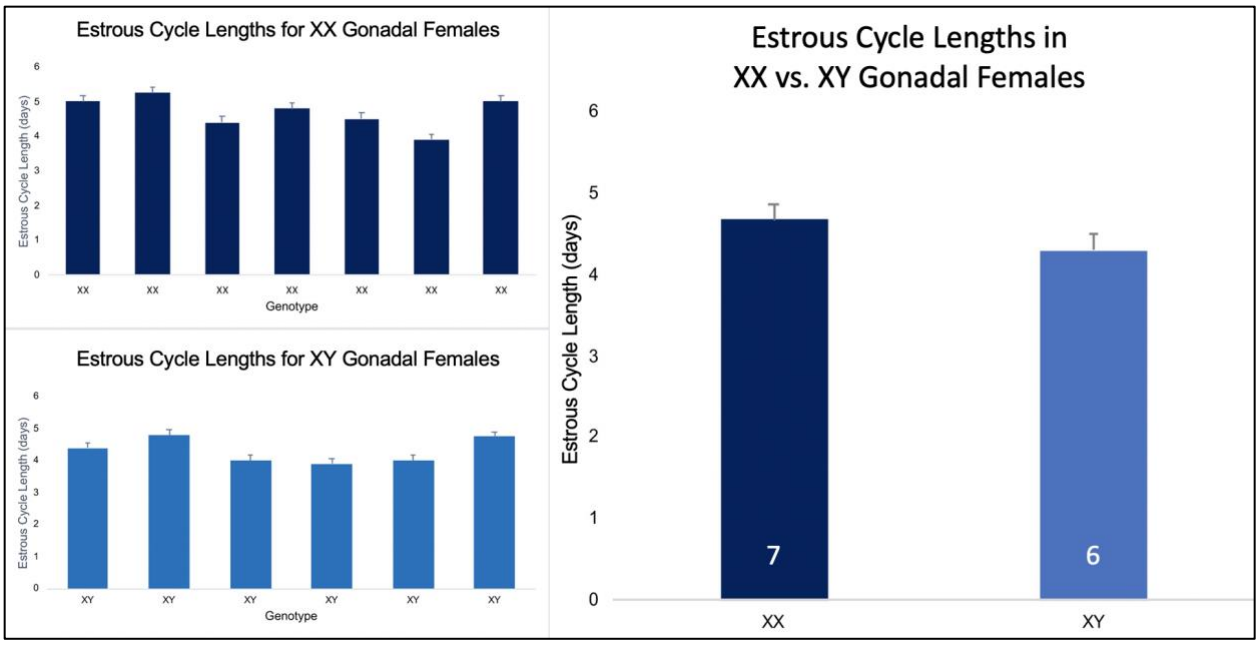

Figure 5: Estrous Cycle Length in gonadal female groups within the FCG rat model. Estrous cycle length was measured for 3-4 cycles in XX (n = 7) and XY$^{\triangle}$ (n = 6) gonadal females using the vaginal cytological patterns described in Figure 4. There were no significant differences found in estrous cycle length for animals within the same group ($p > 0.05$). No significant differences were also observed between the mean estrous cycle length of XX and XY$^{\triangle}$ gonadal females suggesting no sex chromosome effects on estrous cycling. Standard error of the mean is indicated with the error bars.

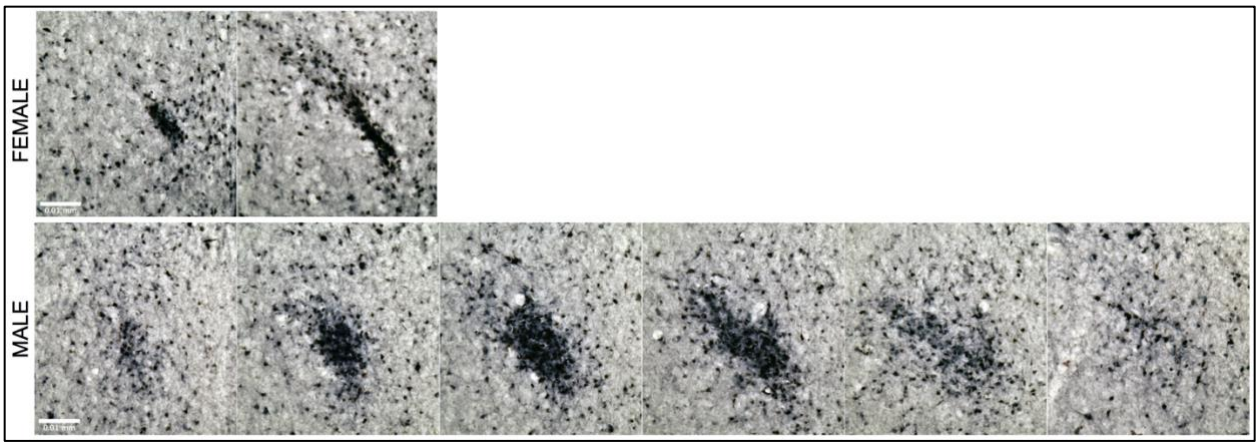

Figure 6: Examples of the series of CALB stained brain sections containing the SDN-POA in gonadal males and gonadal females. Representative images were taken at 20x magnification (scale bar: 0.01 mm) using brightfield microscopy. The SDN-POA was identified using immunohistochemistry to label CALB-ir neurons and identify a border around the nucleus within each section. The SDN-POA was consistently contained within 5-6 sections for gonadal males and 2-3 sections for gonadal females.

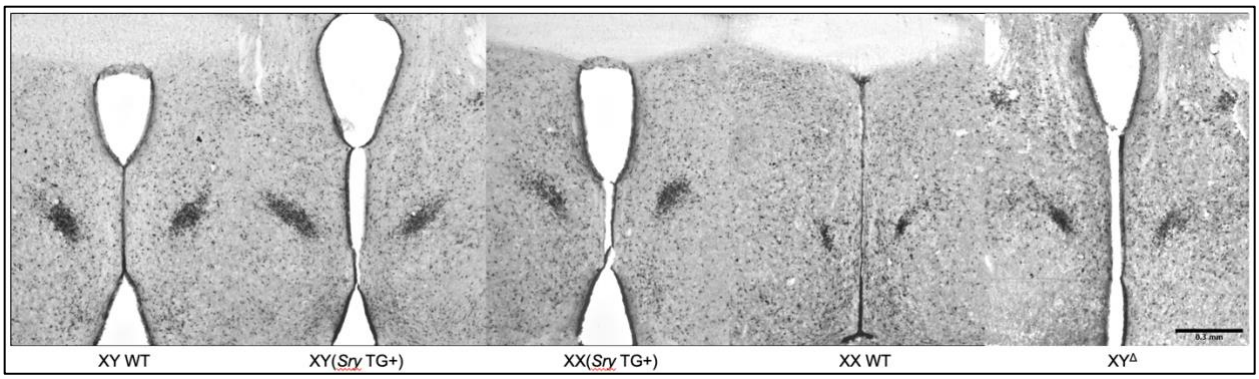

Figure 7: SDN-POA CALB-ir cells in all five genotypes in the Four Core Genotypes rat model. Representative images were taken at 5x magnification (scale bar: 0.3 mm) and depicts the distribution of CALB-ir within the SDN-POA at its maximal area in coronal sections across all five genotypes. The SDN-POA in gonadal males was much larger in size compared to gonadal females, which was later confirmed using a quantitative analysis of cell number, cell size, and nucleus volume.

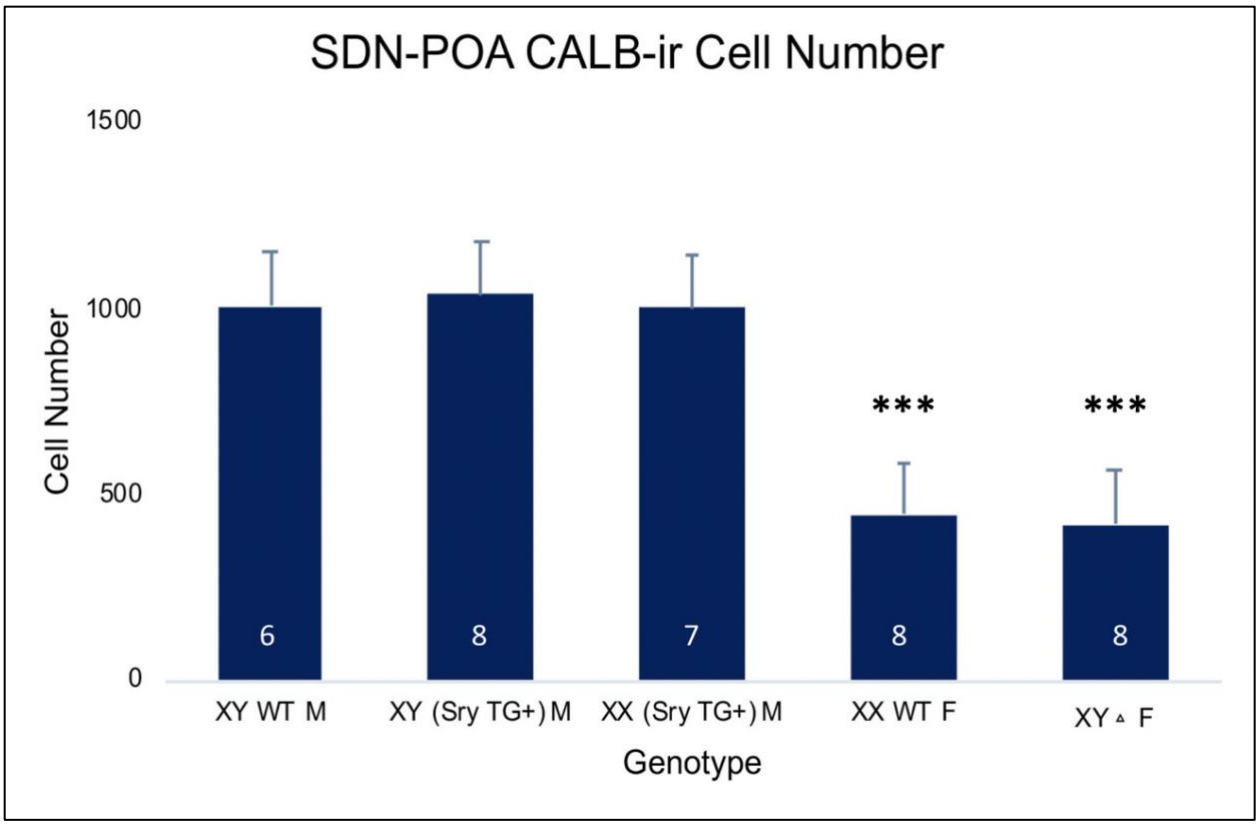

Figure 8: Sex Differences in SDN-POA CALB-ir Cell Number in adult male and female rats using the Four Core Genotypes Rat Model. There was a significant difference in CALB-ir cell number between gonadal male and gonadal female groups. One-way ANOVA showed a significant difference among groups ($p < 0.001$). Post hoc tests further showed that gonadal males all had a larger cell number in the SDN-POA compared to either group of gonadal females ($p < 0.05$). There was no apparent sex chromosome effect on the sex difference in SDN-POA CALB-ir cell number. The graph shows the mean, standard error of the mean, and group size.

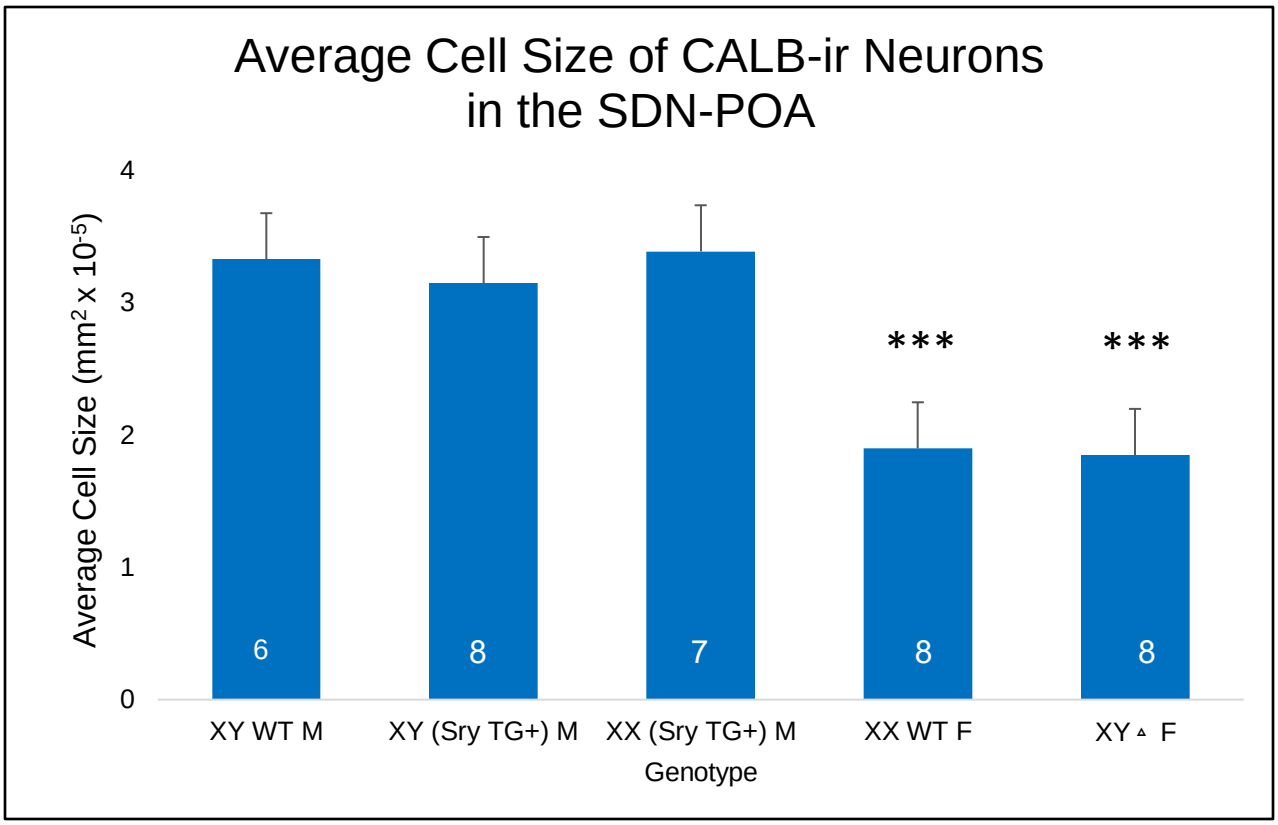

Figure 9: Sex Differences in Average Cell Size of CALB-ir Neurons in the SDN-POA using Four Core Genotypes Rat Model. Cross sectional cell area was significantly different among groups (one-way ANOVA, $p < 0.001$. Posthoc tests showed that each male group differed from each female group ($p < 0.05$). TThere was no apparent sex chromosome effect on the sex differences in the average cell size of CALB-ir neurons in the SDN-POA. The graph shows the mean, standard error of the mean, and group size.

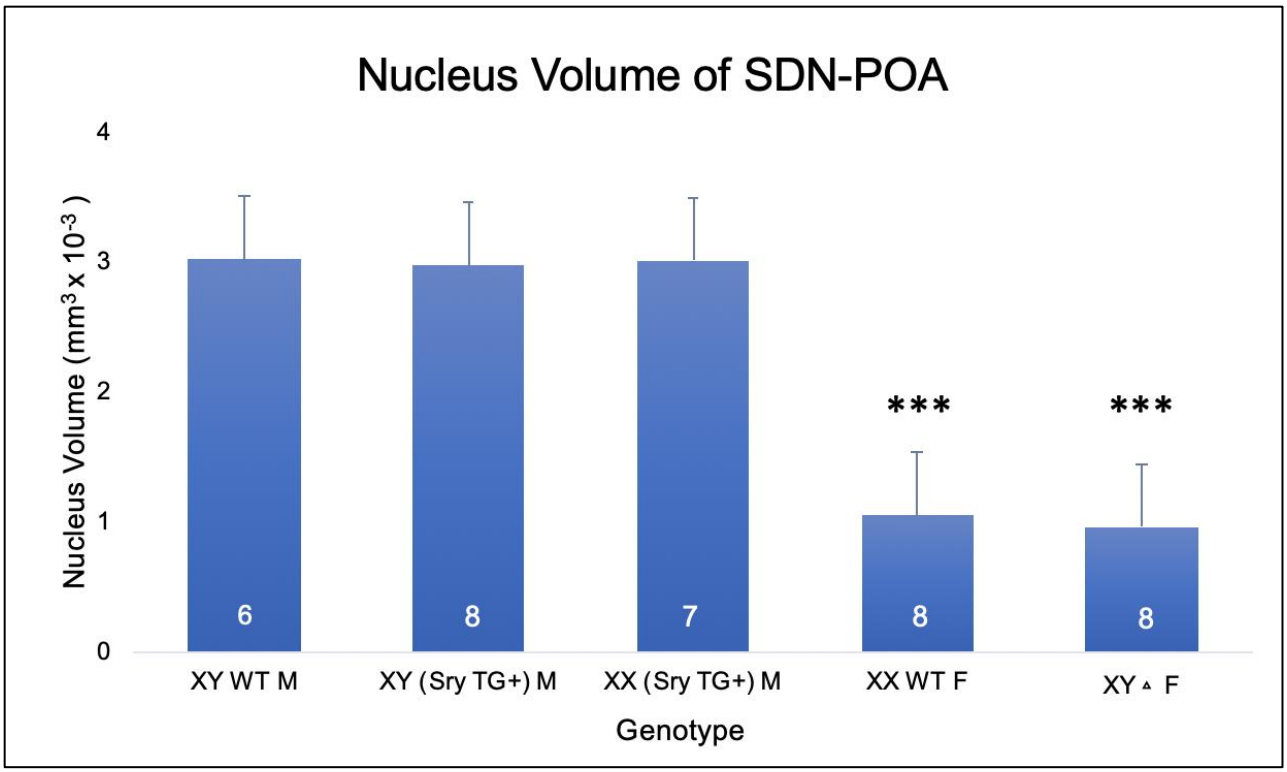

Figure 10: Sex Differences in Nucleus Volume of the SDN-POA using the Four Core Genotypes Rat Model. The overall nucleus volume, quantified using CALB-ir neurons in the SDN-POA, was found to vary significantly among groups (one-way ANOVA, $p < 0.001$). Each gonadal male group had a larger nucleus volume in comparison to each gonadal female group (Tukey-Kramer, $p < 0.05$). There was no apparent sex chromosome effect on the sex differences in overall nucleus volume of the SDN-POA. The graph shows the mean, standard error of the mean, and group size.

TABLES

A

	XX WT (n = 7)	XY$^{\Delta}$ (n = 6)
Estrous Cycle Length (days)	4.69 ± 0.17	4.31 ± 0.16

B

	XY WT (n = 6)	XY(*Sry*TG+) (n = 8)	XX(*Sry*TG+) (n = 7)	XX WT (n = 8)	XY$^{\Delta}$ (n = 8)
Cell Number	1006 ± 42	1036 ± 33	1000 ± 49	441 ± 28	428 ± 26
Cell Size ($mm^2 \times 10^{-5}$)	3.33 ± 0.08	3.15 ± 0.06	3.39 ± 0.10	1.90 ± 0.07	1.85 ± 0.09
Nucleus Volume ($mm^3 \times 10^{-3}$)	3.02 ± 0.17	2.97 ± 0.09	3.01 ± 0.10	1.07 ± 0.12	0.963 ± 0.0.05

Table 1: A. Estrous cycle length in days for XX WT and XY$^{\Delta}$ gonadal females. The reported estrous cycle length in days is an average value of 3-4 consistent estrous cycles. There was no significant difference found in the estrous cycle length between XX and XY$^{\Delta}$ gonadal females ($p > 0.05$) *B. Cell number, cell size, and nucleus volume for the SDN CALB-ir using the five genotypes in the FCG rat model.* All measured variables were found to vary significantly among groups ($p < 0.001$), and to be larger in each male group compared to each female group. There were no significant differences found within each group of the same sex ($p > 0.05$).

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