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Estrogen Receptor Expression In Sleep Regulatory Brain Regions: Effects of Genotypic and
Phenotypic Sex

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Physiological Science

by

Mackenna Kahan

2026

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ABSTRACT OF THE THESIS

Estrogen Receptor Expression In Sleep Regulatory Brain Regions: Effects of Genotypic and Phenotypic Sex

by

Mackenna Kahan

Master of Science in Physiological Science

University of California, Los Angeles, 2026

Professor Ketema Paul, Chair

Sleep disorders are a major public health concern with many known comorbidities, such as heart disease, depression, and anxiety. Women are more susceptible to sleep disorders such as insomnia. Previous studies have shown that males exhibit more resilient recovery phenotypes following sleep disruptions.¹ There is a significant overlap in regulatory brain regions involving sleep and stress, particularly in the hypothalamus. We hypothesize that the resilience to sleep loss typically observed in males is a component of the sleep recovery response following psychological stress. The Four Core Genotype (FCG) is a mouse model that allows us to elucidate sex differences in sleep by separating genotypic and phenotypic aspects of sex. Brain regions responsible for homeostatic mechanisms of sleep and stress will be isolated from the hypothalamus following either one hour of sleep deprivation (GH) or restraint stress (RS). We performed fluorescent in situ hybridization (FISH) to visualize and quantify estrogen receptor α and β expression levels. Our results will contribute to our understanding of the molecular and cellular mechanisms of sex differences in sleep. Our findings demonstrated significant differences in ER α expression were observed within the SCN between genotype and phenotypic sex groups under intact conditions. Following gonadectomy, ER α expression remained significantly altered within the SCN, while significant phenotypic sex differences in both ER α and ER β expression emerged within the VLPO.

The thesis of Mackenna Kahan is approved.

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2026

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Introduction

Sleep Disorders and Sex Differences

Sleep is essential for maintaining cognitive function, emotional regulation, and overall physiological health. Disruptions in sleep are associated with increased risk for psychiatric disorders, metabolic dysfunction, and impaired daily functioning, making sleep disorders a major public health concern [1,13–16]. Most sleep disorders exhibit sex differences and among the most well documented are insomnia [1–3]. Sleep complaints such as insufficient sleep and insomnia are more prevalent in women [2,3]. This sex difference in insomnia emerges after puberty, suggesting that hormonal events underlying puberty may be involved [33–36]. This is the same age that sex differences in mental health disorders become prevalent showing that sex differences in mental health follow a similar pattern to insomnia. The 3 primary causes of insomnia are homeostatic impairments, circadian dysfunction (jet lag, shift work) and anxiety (physiological stress responses). Despite the clear sex differences in insomnia prevalence, the biological mechanisms driving these differences remain incompletely understood. In particular, it is unclear how interactions between hormonal, genetic, and neural regulatory systems contribute to these disparities. This work will focus on the homeostatic impairments seen and will focus on targeting those specific ones in homeostatic mechanisms of sleep regulation.

Two Process Model

Sleep is regulated by both circadian and homeostatic processes, as outlined in the two-process model [17]. The Two-Process Model states that sleep is regulated by the interaction of homeostatic sleep pressure (Process S) and the circadian timing system (Process C) [17]. Process C refers to the circadian process, where an internal clock helps consolidate sleep at specific times of the day. In diurnal species, like humans, sleep typically occurs at night, while in nocturnal species such as mice, sleep happens during the day. Process S, the homeostatic component, reflects the accumulation of sleep pressure during wakefulness and its dissipation during sleep [17]. Disruptions in this process are thought to contribute significantly to insomnia, particularly in cases where individuals are unable to initiate or maintain sleep despite sufficient opportunity. Importantly, key brain regions involved in these processes include the ventrolateral preoptic nucleus (VLPO), which promotes sleep, and the suprachiasmatic nucleus (SCN), which serves as the master circadian clock [5,7]. Understanding how

these systems differ between sexes is critical for identifying mechanisms underlying sex-specific sleep phenotypes.

Sex Differences in Sleep Homeostasis

Although influenced by environmental and psychosocial factors these gender disparities are largely driven by sex differences in sleep regulatory mechanisms. Previous studies have identified sex differences in sleep homeostasis, with female mice exhibiting greater wakefulness and reduced NREM sleep compared with males, as well as a more robust NREM rebound following sleep deprivation when normalized to baseline levels [1,4,18]. Gonadectomy largely eliminates these sex differences in sleep architecture and NREM recovery, indicating a primary role for gonadal hormones[1,9] . However, the dissipation of slow-wave activity (SWA) during recovery sleep occurs more quickly in males than females, even after gonadectomies. These findings suggest that biological sex influences both baseline sleep architecture and the physiological response to sleep loss. However, while gonadal hormones clearly contribute to these differences, they do not fully explain them [4]. Persistent differences following gonadectomy indicate that additional factors, such as sex chromosome complement or early developmental hormone exposure, may also play a role.

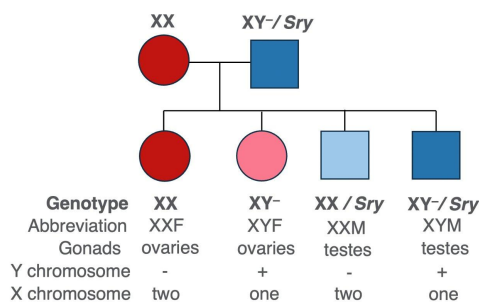
Sex differences in the effects of stress on sleep are driven by gonadal hormones [9]. This was a pivotal finding because it revealed that sex differences in sleep homeostatic mechanisms are generated through different processes than those that drive sleep responses to stress. When it comes to homeostatic mechanism sleep responses, effects of stress are sensitive to the reproductive hormone environment while effects of sleep loss are not. In these experiments we will just be isolating the effects of sleep loss and the homeostatic response in gene expression.

Four Core Genotype Mouse Line

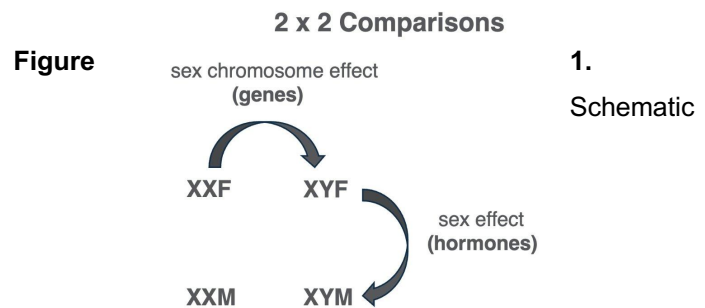
We use the Four Core Genotype mouse line which separates chromosomal sex from gonadal sex, allowing us to compare genetic and hormonal contributions [4]. A major limitation in studying sex differences is the confounding relationship between chromosomal sex and gonadal sex, as they are typically aligned in standard models. In this model, the testis-determining gene Sry is deleted from the Y chromosome and inserted onto an autosome, producing XX and XY mice that can develop either ovaries or testes independently of their sex chromosomes. As a result, the FCG model generates four groups—XX females, XY females, XX males, and XY males—allowing investigators to independently assess the contributions of gonadal hormones and sex chromosome complement to physiological and behavioral

phenotypes [4]. By disentangling these variables, the FCG model allows researchers to determine whether observed sex differences are driven primarily by genetic factors, hormonal influences, or an interaction between the two. This approach has been widely used to clarify the mechanisms underlying sex differences in neural, endocrine, and metabolic regulation. Through this mouse line we are able to make a 2x2 comparison as shown in Figure 2 below. This approach provides a powerful framework for identifying the underlying mechanisms of sex differences in sleep regulation, which cannot be resolved using traditional animal models.

A



B



representation of the Four Core Genotype (FCG) mouse model and comparison strategy used to separate the effects of sex chromosome complement and gonadal phenotype. XXF = XX female, XYF = XY⁻ female, XXM = XX/Sry male, and XYM = XY⁻/Sry male. Diagram adapted from Arnold et al. (2009)

The four-core genotype (FCG) mouse model offers a robust framework for investigating the persistent sex differences following GDX. Mice from the FCG mouse model exhibit no sex differences in rest-phase sleep amount; however, during the active-phase (nighttime), XX males have more spontaneous NREM sleep than XX females. The XY male and female mice do not exhibit sex differences in sleep amount. During rebound from forced wakefulness, there is a change in the factors regulating sleep. XY females sleep more during their mid-active phase than XX females and have higher NREM SWA [10]. These findings suggest that processes that regulate sleep homeostasis are sex-linked, and that sleep amount and sleep propensity are regulated differently in males and females during rebound from sleep loss.

The Role Estrogen Plays

We focus on estrogen receptors because they are highly expressed in key brain regions involved in sleep regulation, such as the VLPO and SCN, and estrogen has been strongly

associated with the sex differences we observe in sleep [5,7,38]. Estrogen activity in VLPO is responsible for sex differences in immediate early gene expression (Fos) in female mice after sleep deprivation [9]. Estrogen receptor expression in the SCN mediates sex differences in circadian phenotypes [5,7]. Estrogen and progesterone have long been implicated in the regulation of sleep architecture and arousal states. Early studies demonstrated that fluctuations in ovarian hormones alter sleep patterns in female rats, providing evidence that reproductive hormones directly modulate sleep behavior. Estradiol was associated with increased wakefulness and altered sleep organization, whereas progesterone produced opposing, sleep-promoting effects [37]. Together, these findings support the idea that dynamic hormonal environments contribute significantly to sex differences in sleep regulation and vulnerability to insomnia.

The production and release of androgens and estrogens in mammals follow a pattern during early development. While the levels of these hormones remain relatively stable during certain periods, there are also sudden and significant changes in both the amount and timing of their secretion throughout development. These changes are key features of growth and maturation. Estrogens such as estradiol and androgens such as testosterone both contribute to sleep quantity and quality; estradiol is associated with wakefulness and sleep consolidation while lowered testosterone levels are associated with poor sleep quality, increased sleep fragmentation, decreased sleep amount and decreased time spent in slow wave sleep [8,10,11,37,38]. Testosterone serves as a precursor for estrogens and is converted into estradiol via the action of aromatase. This conversion is a primary mechanism driving sexual differentiation of neural circuits [19–31]. Consistent with this, estradiol was shown to produce a stronger masculinizing effect than dihydrotestosterone on vasopressin expression in the BST, highlighting the importance of estrogen receptor signaling when examining sex differences in receptor expression[20].

Estrogen has been shown to increase gene transcription and neuronal excitability, leading to enhanced synaptic activity and behaviorally relevant neural output. Progesterone amplifies and refines these estrogen-driven effects by modulating gene expression and cellular signaling pathways [24,37,38]. These interactions illustrate how hormone-dependent mechanisms can link individual gene activity to complex social behaviors.

Estrogen reduces total sleep and promotes wakefulness, while progesterone reverses these effects by increasing sleep and decreasing arousal [37,38]. Together, these findings demonstrate that sleep architecture is dynamically regulated by interacting with gonadal hormones. In addition to reproductive physiology, gonadal hormones strongly influence neural systems involved in arousal, motivation, and behavior. Previous studies proposed that hormonal and genetic mechanisms interact to regulate arousal-related neural circuitry underlying sex-specific behaviors [24]. Because sleep and arousal systems are tightly interconnected, these findings further support the hypothesis that estrogen receptor signaling may contribute to sex differences in sleep homeostasis and circadian function.

Gonadal hormones are hormones produced by the ovaries in females and testes in males, they play a crucial role in sexual development, reproduction and regulation of secondary sex traits. Gonadal hormones have two primary effects on the brain and secondary sex traits: organizational and activational. In rodents, the hormone environment during birth plays a crucial role in "organizing" brain circuits that later control sex-specific behaviors, such as mating, aggression, arousal and stress responses [19,21–32]. The presence of androgens in males, or their absence in females, a couple days after birth are a critical period that helps shape brain and behavior functions that are specific to each sex [19,21]. The sex-specific behaviors that we see later in life, depend on how these early hormonal influences organize the brain and behavior.

Puberty is defined as the period when reproduction becomes possible for the first time [33–36]. While sexual maturity (the time when reproductive capacity is at its peak) can develop gradually over a longer period, puberty marks a change in the hormones that control reproduction and influence sex-specific behaviors. The hormonal changes that occur during puberty are similar across mammal species, but the timing of these events can vary greatly between and even within species. In male mice, the testes begin to descend around 21 days of age [36]. In female mice, puberty is marked by vaginal opening, which happens at around 27 days of age [34].. The process of puberty is gradual, and our understanding of how adult sleep patterns, which differ by sex, develop before, during, or after puberty is still limited.

Hypothesis

The present study aims to investigate the independent and interactive effects of sex chromosome complement and gonadal sex on estrogen receptor expression in key brain regions involved in sleep and circadian regulation. Sex chromosome complement and gonadal sex contribute to differences in estrogen receptor (ER α and ER β) expression within sleep- and circadian-regulating brain regions. Specifically, phenotypic males (XYM, XXM) will exhibit different levels of estrogen receptor expression in the ventrolateral preoptic nucleus (VLPO) and suprachiasmatic nucleus (SCN) compared to phenotypic females (XYF, XXF).

To test this overarching hypothesis, a series of experiments were conducted using the Four Core Genotypes (FCG) mouse model to dissociate the effects of sex chromosomes and gonadal sex. First, ER α and ER β expression were measured in gonadally intact animals to determine whether baseline differences exist across both phenotypic sex and genotype. It is expected that phenotypic males (XYM, XXM) will exhibit different ER α and ER β mRNA expression patterns within the SCN and VLPO compared with phenotypic females (XYF, XXF). Next, to examine the role of pubertal gonadal hormones in organizing these differences, mice underwent pre-pubertal gonadectomy (GDX). Following gonadectomy, it is predicted that phenotypic males (XYM, XXM) will exhibit altered estrogen receptor expression in the VLPO and SCN compared to phenotypic females (XYF, XXF), indicating that circulating hormones during development contribute to sex-specific patterns of ER expression.

Methods

Animals

All animal procedures were conducted in accordance with the guidelines established by the UCLA Division of Laboratory Animal Medicine and approved institutional protocols. The Four Core Genotypes (FCG) mouse model was used to dissociate the effects of sex chromosome complement (XX vs. XY) from gonadal sex (testes vs. ovaries). Mice were bred and maintained under standard laboratory conditions and group-housed prior to experimental manipulation. Animals were maintained on a 12 h light:12 h dark (LD) cycle, with lights on at Zeitgeber Time (ZT) 0 and off at ZT12. Ambient temperature and humidity were controlled, and food and water

were available ad libitum. All mice were acclimated to housing conditions and handling prior to experimentation to minimize stress-induced variability. Experimental cohorts consisted of $n = 3$ animals per genotype unless otherwise specified. For the purposes of this study, genotype refers to sex chromosome complement (XX vs. XY), phenotypic sex refers to gonadal phenotype (testes vs. ovaries), and gonadal status refers to whether animals were gonadally intact or gonadectomized (GDX). Phenotype-based analyses were conducted independent of genotype, whereas genotype-based analyses were conducted independent of phenotypic sex.

Experimental Design

Two complementary experimental paradigms were used to investigate the role of sex chromosome complement and pubertal gonadal hormones in regulating estrogen receptor expression.

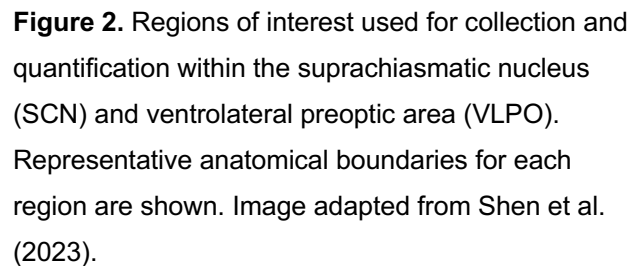
In Experiment 1a, gonadally intact FCG mice were used to assess baseline differences in estrogen receptor alpha ($ER\alpha$) and estrogen receptor beta ($ER\beta$) expression across genotypes and phenotypic sexes. Mice were gently handled for approximately 1 hour prior to sacrifice to reduce acute stress responses. Animals were sacrificed during the mid-rest phase to control circadian variation in gene expression.

In Experiment 1b, mice underwent pre-pubertal gonadectomy (GDX) at postnatal day (PND) 21 to eliminate the organizational effects of endogenous gonadal hormones during development. At adulthood, animals were sacrificed at ZT6 (mid-inactive phase) via cervical dislocation. The use of anesthetics was avoided to prevent acute alterations in transcriptional activity.

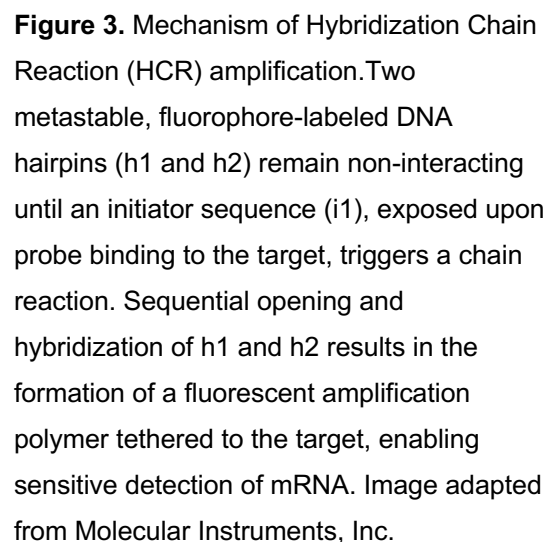
Tissue Collection and Processing

Immediately following sacrifice, brains were rapidly extracted and snap frozen in isopentane chilled with liquid nitrogen to preserve RNA integrity. Frozen samples were wrapped in aluminum foil and stored at -80°C until further processing.

appropriately until in situ hybridization was performed.



Fluorescent in situ hybridization (FISH) was used to detect and localize ER α and ER β mRNA expression within brain tissue. This method relies on the hybridization of labeled nucleotide probes to complementary RNA sequences, enabling precise spatial mapping of gene expression within defined brain regions.



Fluorescently labeled probes specific to ER α and ER β were applied to tissue sections under controlled hybridization conditions. Following incubation, sections were subjected to a series of washes to remove nonspecific binding. Slides were then coverslipped using an antifade mounting medium to preserve fluorescence signal. This approach allowed for visualization of receptor-specific punctate signals within the VLPO and SCN at cellular resolution.

Image Acquisition

Fluorescent images were acquired using a fluorescence microscope at 20 $\times$ magnification. Imaging parameters, including exposure time, gain, and illumination intensity, were held constant across all samples to ensure comparability between experimental groups.

Regions of interest (ROIs) corresponding to the VLPO and SCN were identified using anatomical landmarks, and images were captured in a consistent manner across animals. For each subject, at least 3 sections per region were imaged, yielding a total of 9 images per animal. Care was taken to avoid oversaturation and to maintain signal within a linear detection range.

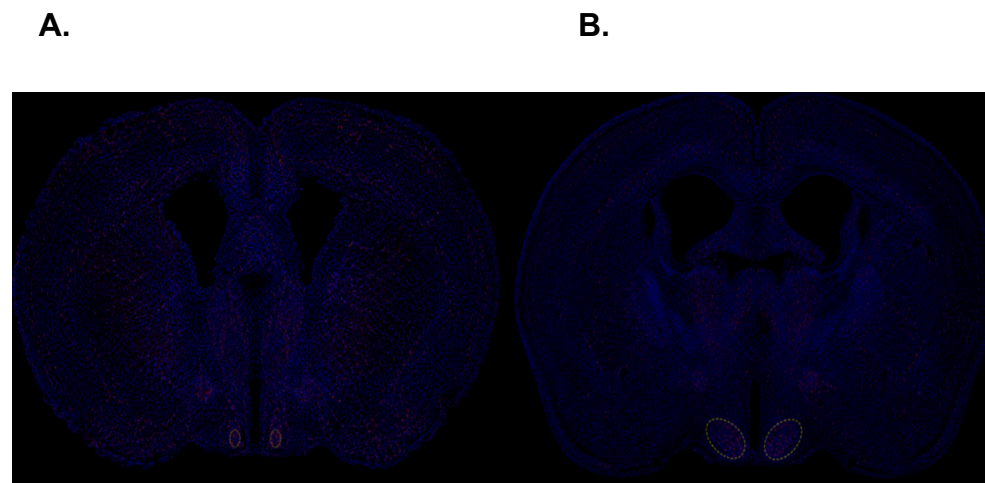


Figure 4A. Image of a coronal brain section showing the ventrolateral preoptic nucleus (VLPO). **4B.** Image of a coronal brain section showing the suprachiasmatic nucleus (SCN). The yellow dashed circles indicate the regions of interest used for image quantification. DAPI-stained nuclei are shown in blue

A.

B.

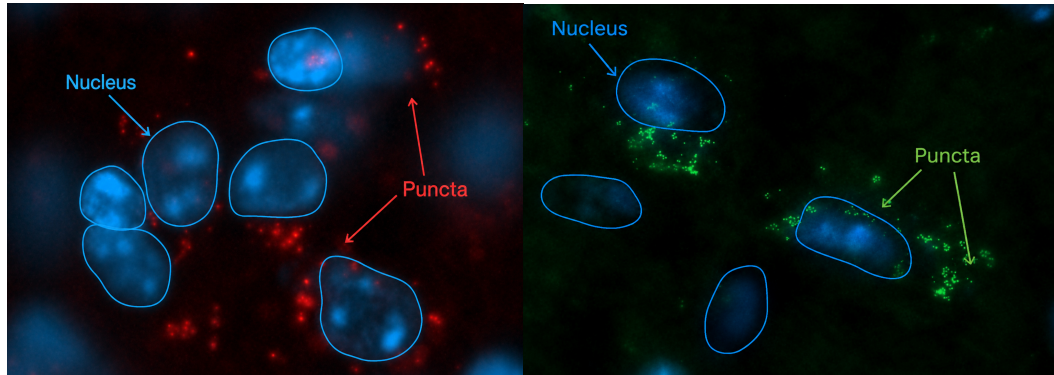


Figure 5A. Image of the suprachiasmatic nucleus (SCN) showing nuclei stained with DAPI (blue) and estrogen receptor α (ER α) expression (red) **5B.** Image of the SCN showing nuclei stained with DAPI (blue) and estrogen receptor β (ER β) expression (green). The circle illustrates nuclei and around it are the puncta counted.

A.

B.

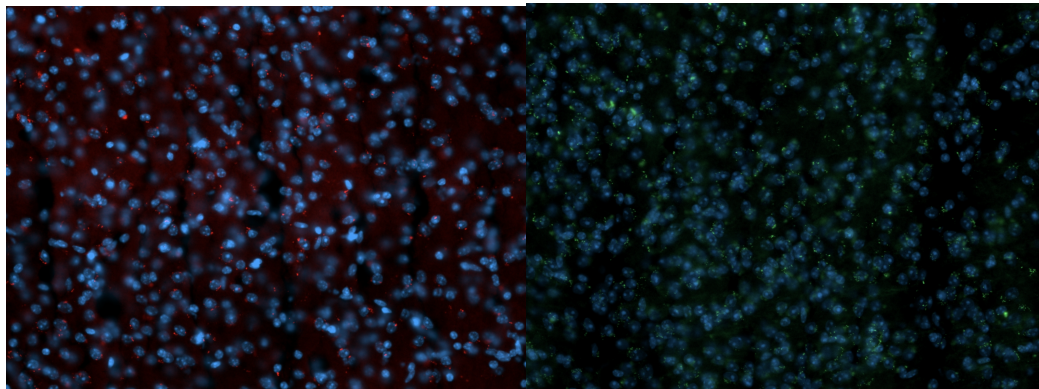


Figure 6A. Image of the suprachiasmatic nucleus (SCN) showing nuclei stained with DAPI (blue) and estrogen receptor α (ER α) expression (red). **Figure 6B.** Image of the SCN showing nuclei stained with DAPI (blue) and estrogen receptor β (ER β) expression (green). All images used 40x magnification.

Quantitative Analysis of ER Expression

Quantitative analysis of ER α and ER β expression was conducted using ImageJ software. All image processing and quantification were performed by an investigator blinded to experimental condition and genotype to minimize observer bias.

Multichannel fluorescent images were separated into individual color channels prior to analysis. The green channel was used for ER β puncta quantification, while the red channel was used for ER α . Background subtraction was applied using a rolling ball algorithm with a radius of 3 pixels to reduce local background variation and enhance detection of punctate signals.

To ensure consistency across samples, a constant global threshold was established for each receptor based on visual optimization of puncta separation and background suppression, and applied uniformly to all images. For ER β , a fixed threshold range of 40-255 was used, and for ER α , a fixed threshold range of 35-255 was applied.

Following thresholding, binary masks were generated and analyzed using the “Analyze Particles” function. Inclusion criteria were defined to exclude noise and non-specific signal: particles were required to fall within a size range of 2–50 pixels² and exhibit a circularity between 0.2 and 1.0.

Puncta counts were obtained for each ROI (VLPO and SCN) and averaged across 3 sections per animal to generate a single representative value per region for statistical analysis.

Statistical Analysis

Following image quantification, puncta count data were compiled and organized using GraphPad Prism for statistical analysis. Statistical analyses were performed to evaluate differences in ER α and ER β expression across genotype, phenotypic sex, and gonadal condition groups.

For comparisons involving multiple independent variables, two-way analysis of variance (ANOVA) was used to assess the main effects of genotype and gonadal condition, as well as their interaction. When significant main effects or interactions were detected, post hoc pairwise comparisons were performed using unpaired two-tailed t-tests. Welch’s correction was applied when variance between groups was unequal. For phenotype-based analyses, animals were grouped according to gonadal phenotype independent of chromosomal sex. For genotype-based analyses, animals were grouped according to sex chromosome complement independent of gonadal phenotype. Statistical significance was defined as $p < 0.05$, $p < 0.01$, and $p < 0.001$. Data are presented as mean $\pm$ SEM.

Results

Estrogen receptor expression in the VLPO and SCN of intact and gonadectomized FCG mice.

To determine whether gonadal status influenced estrogen receptor expression in sleep- and circadian-regulating brain regions, ER α and ER β puncta counts were quantified in the VLPO and SCN of intact and gonadectomized FCG mice (Fig. 1). Representative quantification revealed detectable ER α and ER β expression across all experimental groups. Overall, gonadectomized animals exhibited altered receptor expression patterns compared to intact mice, although the magnitude of these effects varied by receptor type and brain region. Significant differences were observed in select comparisons, while other receptor populations remained unchanged between groups. Together, these findings suggest that both gonadal hormones and sex chromosome complement contribute to estrogen receptor expression within hypothalamic sleep regulatory regions.

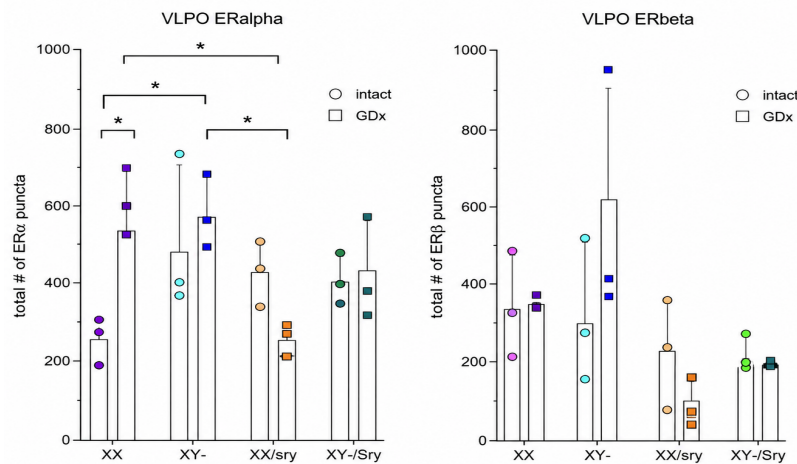


Figure 7. ER α and ER β expression in the VLPO of intact and gonadectomized FCG mice.

Quantification of ER α and ER β puncta counts in the ventrolateral preoptic nucleus (VLPO) and suprachiasmatic nucleus (SCN) across Four Core Genotype (FCG) mice. White bars represent intact mice and gray bars represent gonadectomized (GDx) mice. Data are presented as mean puncta count $\pm$ SEM. Statistical significance was determined using two-way ANOVA followed by post hoc comparisons where appropriate. *p < 0.05, **p < 0.01.

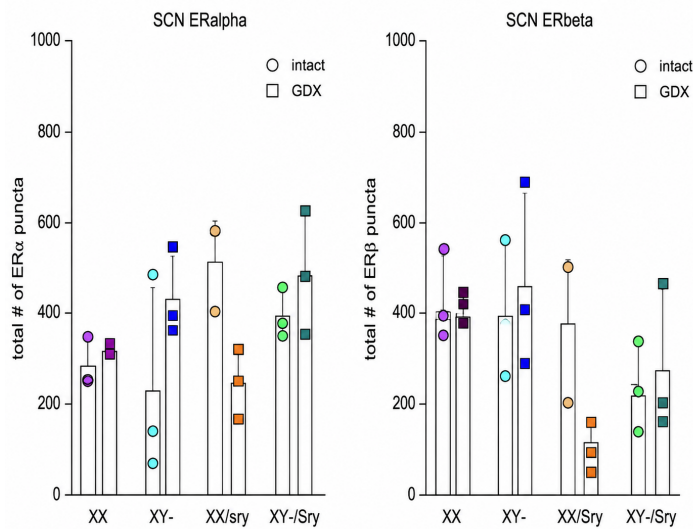


Figure 8. ER α and ER β expression in the SCN of intact and gonadectomized FCG mice.

Quantification of ER α and ER β puncta counts in the ventrolateral preoptic nucleus (VLPO) and suprachiasmatic nucleus (SCN) across Four Core Genotype (FCG) mice. White bars represent intact mice and gray bars represent gonadectomized (GDX) mice. Data are presented as mean puncta count $\pm$ SEM. Statistical significance was determined using two-way ANOVA followed by post hoc comparisons where appropriate. * $p < 0.05$, ** $p < 0.01$.

Intact FCG Mice: Genotype Comparisons

To investigate the effects of sex chromosome complement on estrogen receptor expression, intact FCG mice were grouped by genotype (XX vs. XY) independent of gonadal phenotype and analyzed for ER α and ER β expression within the SCN and VLPO (Fig. 9-10). Within the SCN, ER α and ER β expression differed slightly between genotypes, with XX mice exhibiting greater puncta counts compared to XY mice (Fig. 9). Within the VLPO, neither ER α nor ER β expression differed between XX and XY mice (Fig. 10), although less variability across animals was observed. Together, these findings suggest that sex chromosome complement could selectively influence ER α expression within the SCN under intact hormonal conditions.

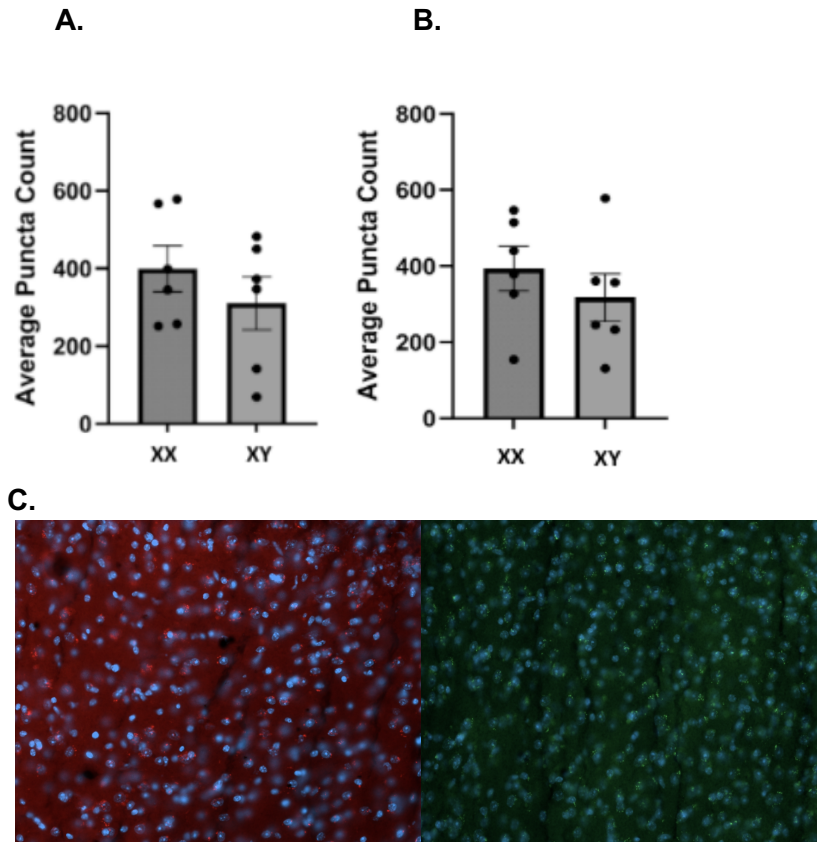


Figure 9. SCN ER α (A) and ER β (B) puncta counts in intact Four Core Genotype (FCG) mice grouped by genotype (XX vs. XY). For genotype-based analyses, the “XX” group included XX and XX/Sry mice, while the “XY” group included XY⁻ and XY⁻/Sry mice. Quantification of estrogen receptor expression within the suprachiasmatic nucleus (SCN) was performed using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. * $p < 0.05$. P value of graph A is .350 P value, graph B is .391. C. Representative fluorescent in situ hybridization (FISH) images corresponding to the quantified data shown in the graphs.

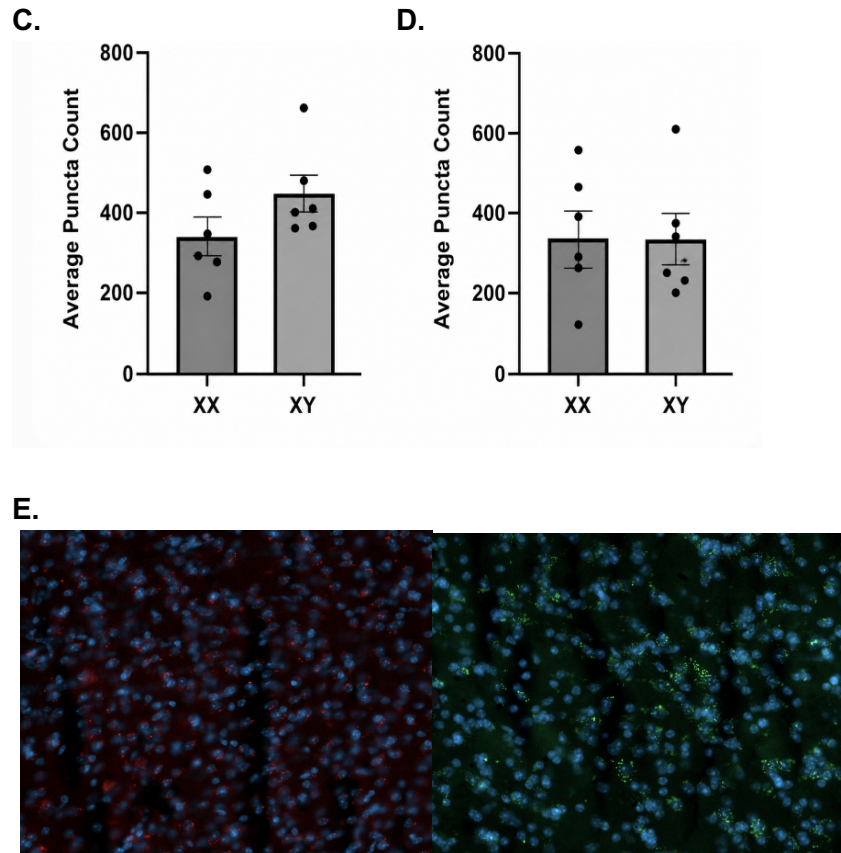


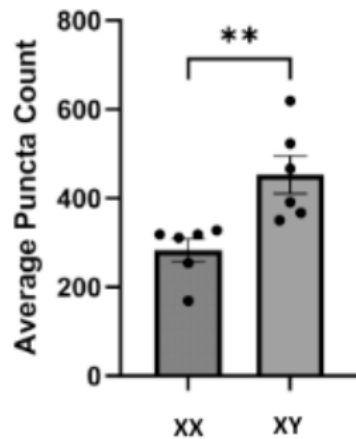
Figure 10. VLPO ERα (C) and ERβ (D) puncta counts in intact Four Core Genotype (FCG) mice grouped by genotype (XX vs. XY). For genotype-based analyses, the “XX” group included XX and XX/Sry mice, while the “XY” group included XY⁻ and XY⁻/Sry mice. Quantification of estrogen receptor expression within the ventrolateral preoptic nucleus (VLPO) was performed using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count ± SEM. P value graph A is .129 P value graph B is .958. E. Representative fluorescent in situ hybridization (FISH) images corresponding to the quantified data shown in the graphs.

GDX FCG Mice: Genotype Comparisons

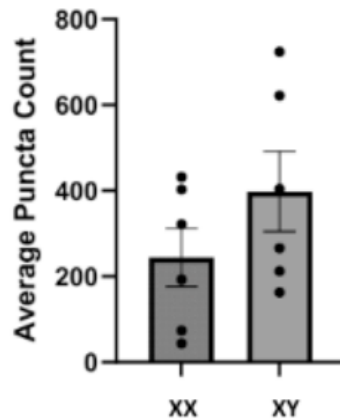
To determine whether removal of gonadal hormones altered genotype-dependent receptor expression, gonadectomized FCG mice were grouped by genotype (XX vs. XY). Within the SCN, ERα puncta counts were significantly elevated in XY mice compared to XX mice following gonadectomy (Fig.11A). No significant genotype differences were detected for SCN ERβ expression (Fig. 11B). Similarly, no significant differences in ERα or ERβ expression were observed within the VLPO between XX and XY gonadectomized mice (Fig.12), although

increased variability was present among XY animals. These findings suggest that gonadal hormone removal alters genotype-dependent ER α expression specifically within the SCN.

A.



B.



C.

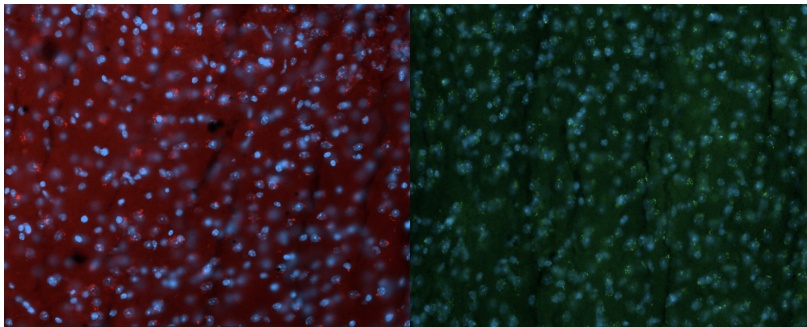


Figure 11. SCN ER α (A) and ER β (B) puncta counts in gonadectomized (GDX) Four Core Genotype (FCG) mice grouped by genotype (XX vs. XY). For genotype-based analyses, the “XX” group included XX and XX/Sry mice, while the “XY” group included XY⁻ and XY⁻/Sry mice. Estrogen receptor expression within the suprachiasmatic nucleus (SCN) was quantified using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. ** $p < 0.01$. P value graph A is .009 P value graph B is .216. C. Representative fluorescent in situ hybridization (FISH) images corresponding to the quantified data shown in the graphs.

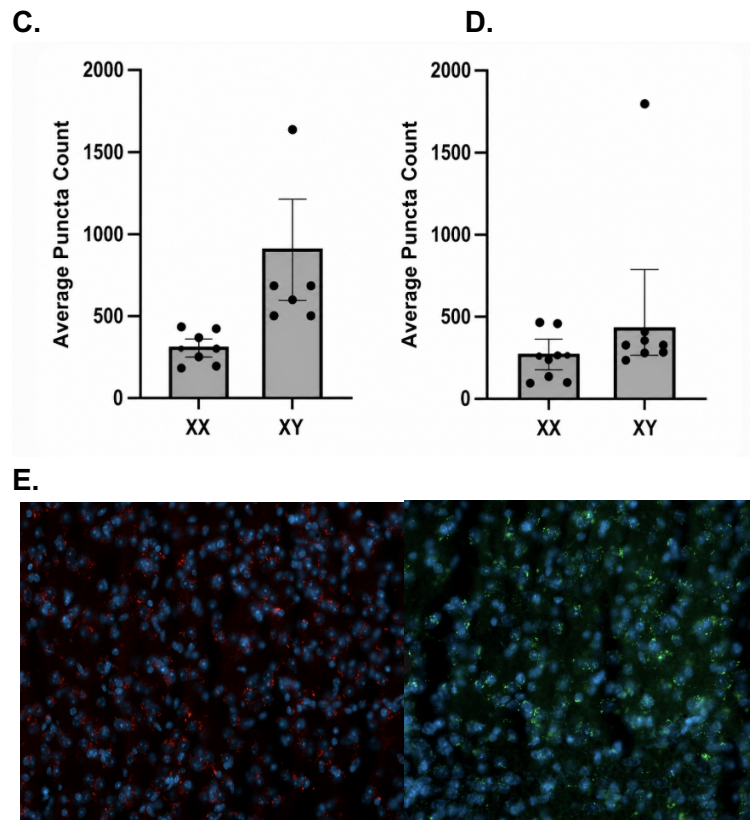


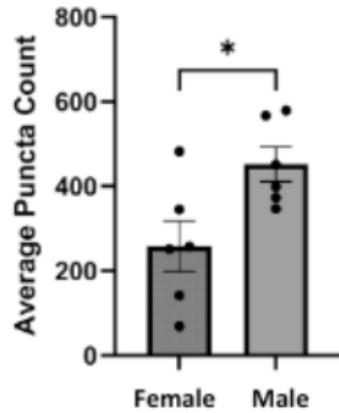
Figure 12. VLPO ER α (C) and ER β (D) puncta counts in gonadectomized (GDX) Four Core Genotype (FCG) mice grouped by genotype (XX vs. XY). For genotype-based analyses, the “XX” group included XX and XX/Sry mice, while the “XY” group included XY⁻ and XY⁻/Sry mice. Estrogen receptor expression within the ventrolateral preoptic nucleus (VLPO) was quantified using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. P value graph A is .284 P value graph B is .257. E. Representative fluorescent in situ hybridization (FISH) images corresponding to the quantified data shown in the graphs.

Intact FCG Mice: Phenotype Comparisons

To evaluate the contribution of phenotypic sex to estrogen receptor expression, intact FCG mice were grouped by gonadal phenotype independent of genotype and analyzed for ER α and ER β expression within the SCN and VLPO (Fig. 13-14). Within the SCN, phenotypic females exhibited significantly greater ER α puncta counts compared to phenotypic males (Fig. 13A). However, no significant phenotypic sex differences were detected for SCN ER β expression (Fig. 13B). Within the VLPO, neither ER α nor ER β expression significantly differed between phenotypic males and females (Fig. 14). Together, these findings suggest that gonadal

phenotype selectively influences ER α expression within the SCN under intact hormonal conditions.

A.



B.

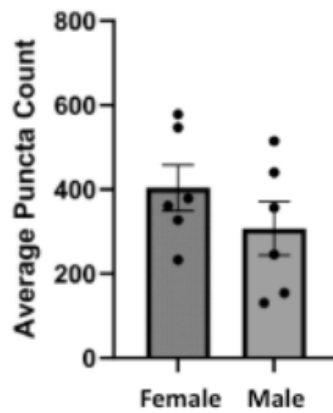
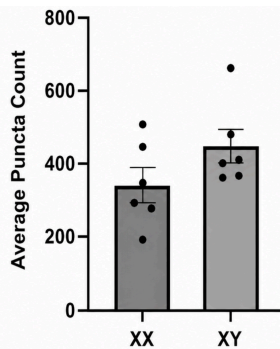


Figure 13. SCN ER α (A) and ER β (B) puncta counts in intact Four Core Genotype (FCG) mice grouped by phenotypic sex (male vs. female). For phenotype-based analyses, the female group consisted of XX and XY⁻ mice, while the male group consisted of XX/Sry and XY⁻/Sry mice. Estrogen receptor expression within the suprachiasmatic nucleus (SCN) was quantified using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. *p < 0.05. P value graph A is .025 P value graph B is .391.

C.



D.

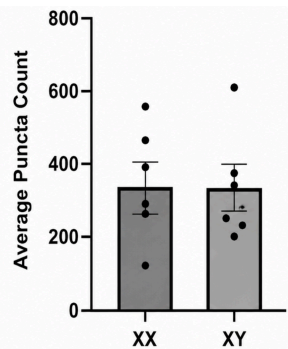


Figure 14. VLPO ER α (C) and ER β (D) puncta counts in intact Four Core Genotype (FCG) mice grouped by phenotypic sex (male vs. female). For phenotype-based analyses, the female group consisted of XX and XY⁻ mice, while the male group consisted of XX/Sry and XY⁻/Sry mice. Estrogen receptor expression within the ventrolateral preoptic nucleus (VLPO) was quantified using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. P value graph A is .5204 P value graph B is .200.

GDX FCG Mice: Phenotype Comparisons

To determine whether phenotypic sex differences in estrogen receptor expression persisted following gonadectomy, GDX FCG mice were grouped by phenotypic sex and analyzed for ER α and ER β expression within the SCN and VLPO (Fig. 15-16). No significant differences in ER α or ER β expression were detected within the SCN between phenotypic males and females following gonadectomy (Fig. 15). In contrast, significant phenotypic sex differences emerged within the VLPO, where gonadectomized phenotypic males exhibited significantly greater ER α and ER β puncta counts compared to gonadectomized phenotypic females (Fig. 16). These findings suggest that gonadal hormone removal may differentially alter estrogen receptor expression within the VLPO.

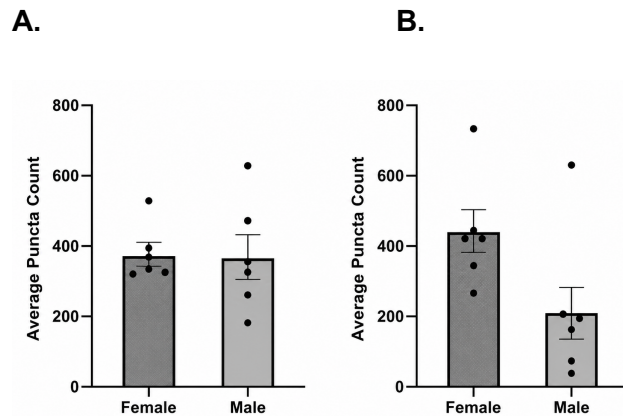


Figure 15. SCN ER α (A) and ER β (B) puncta counts in gonadectomized (GDX) Four Core Genotype (FCG) mice grouped by phenotypic sex (male vs. female). For phenotype-based analyses, the female group consisted of XX and XY⁻ mice, while the male group consisted of XX/Sry and XY⁻/Sry mice. Estrogen receptor expression within the suprachiasmatic nucleus (SCN) was quantified using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. P value graph A is .025. P value graph B is .083.

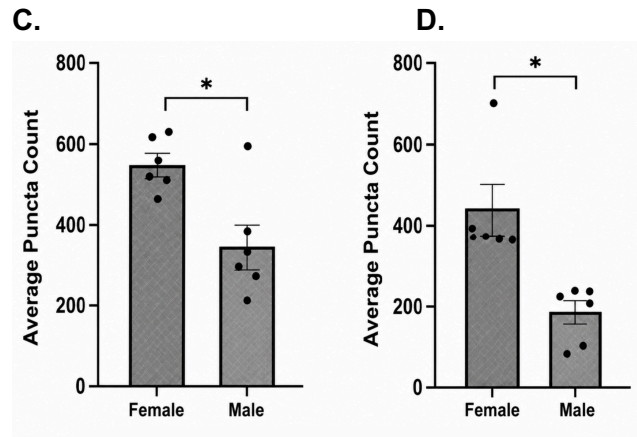


Figure 16. VLPO ER α (C) and ER β (D) puncta counts in gonadectomized (GDX) Four Core Genotype (FCG) mice grouped by phenotypic sex (male vs. female). For phenotype-based analyses, the female group consisted of XX and XY⁻ mice, while the male group consisted of XX/Sry and XY⁻/Sry mice. Estrogen receptor expression within the ventrolateral preoptic nucleus (VLPO) was quantified using fluorescent in situ hybridization (FISH). Data are presented as mean puncta count $\pm$ SEM. * $p < 0.05$. P value graph A is .0133. P value graph B is .0245.

Intact vs GDX Mice: Phenotype and Genotype Comparisons

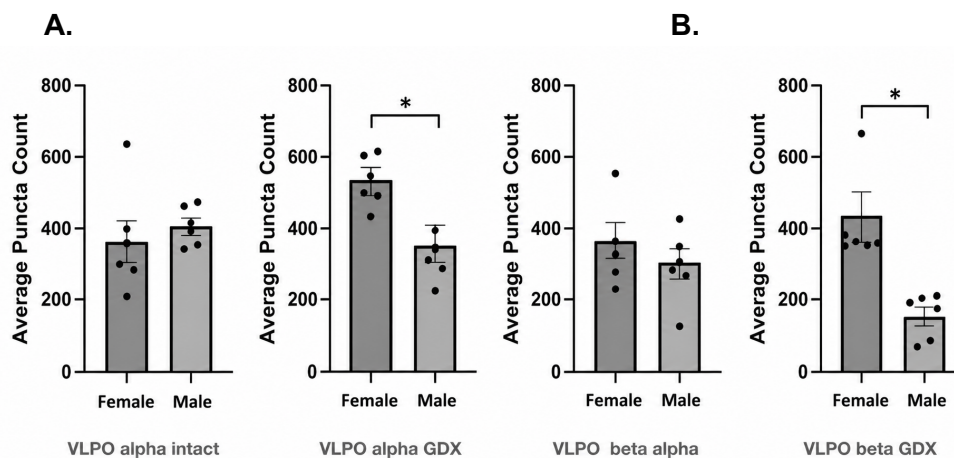
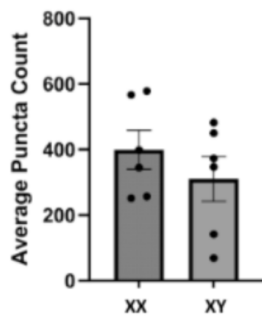


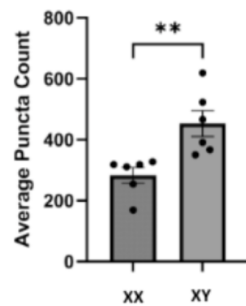
Figure 17. Comparison of estrogen receptor expression in the ventrolateral preoptic nucleus (VLPO) between intact and gonadectomized mice across phenotypes. (A) Estrogen receptor α (ER α) expression in the VLPO of intact and gonadectomized mice. (B) Estrogen receptor β (ER β) expression in the VLPO of intact and gonadectomized mice. Data are presented by phenotype, and statistical comparisons were performed to evaluate the effects of gonadal status on receptor expression within the VLPO. Error bars represent $\pm$ SEM. $p < 0.05$.

A.

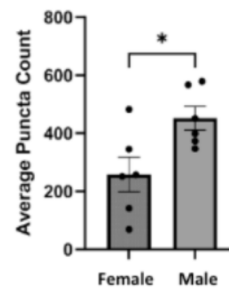


SCN alpha intact

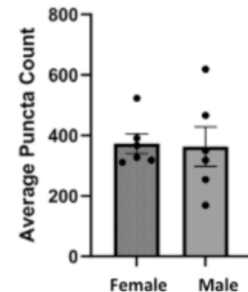
B.



SCN alpha GDX



SCN alpha intact (phenotype)



SCN alpha GDX (phenotype)

Figure 18. Comparison of estrogen receptor expression in the suprachiasmatic nucleus (SCN) between intact and gonadectomized mice across genotypes and phenotypes. (A) Estrogen receptor α (ER α) expression in the SCN of intact and gonadectomized mice. (B) Estrogen receptor β (ER β) expression in the SCN of intact and gonadectomized mice. Data are presented by genotype and phenotype, and statistical comparisons were performed to assess the effects of gonadal status, genotype, and their interaction on receptor expression within the SCN. Error bars represent $\pm$ SEM. $p < 0.05$.

Discussion

Results Summary

The present study investigated the independent and interactive effects of sex chromosome complement and gonadal phenotype on estrogen receptor expression within sleep- and circadian-regulating brain regions using the Four Core Genotype (FCG) mouse model. Specifically, ER α and ER β expression were quantified within the ventrolateral preoptic nucleus (VLPO) and suprachiasmatic nucleus (SCN) in both intact and gonadectomized (GDX) animals. Overall, the findings demonstrate that estrogen receptor expression is differentially influenced by both genotype and phenotypic sex depending on brain region and hormonal status. Within intact animals, sex chromosome complement appeared to selectively influence ER α expression within the SCN. In contrast, ER β expression within the SCN did not significantly differ by genotype. Similarly, neither ER α nor ER β expression within the VLPO significantly differed between XX and XY mice under intact conditions.

These findings suggest that chromosomal sex may contribute more strongly to ER α signaling within circadian regulatory regions than within sleep-promoting nuclei under baseline hormonal conditions. Phenotypic sex comparisons in intact animals revealed a similar pattern. Phenotypic females exhibited significantly greater ER α expression within the SCN compared to phenotypic males, while ER β expression remained unchanged. Within the VLPO, no significant phenotypic sex differences were observed for either receptor subtype. Together, these results suggest that under intact hormonal conditions, estrogen receptor expression differences are more prominent within the SCN than the VLPO, particularly for ER α .

Following gonadectomy, receptor expression patterns shifted considerably. Genotype comparisons in GDX animals demonstrated significantly elevated ER α expression within the SCN of XY mice compared to XX mice, suggesting that removal of gonadal hormones alters the relationship between sex chromosome complement and estrogen receptor signaling. However, ER β expression within the SCN remained unaffected by genotype. Similarly, no significant genotype-dependent differences were observed within the VLPO following gonadectomy, although variability among animals increased.

Interestingly, phenotypic sex comparisons following gonadectomy revealed an opposite regional pattern compared to intact animals. While no significant sex differences were detected within the SCN, gonadectomized phenotypic females exhibited significantly greater ER α and ER β expression within the VLPO compared to gonadectomized phenotypic males. These findings suggest that removal of gonadal hormones may unmask latent sex-dependent differences in estrogen receptor expression within sleep-promoting regions of the hypothalamus. Collectively, these findings support the hypothesis that both sex chromosome complement and gonadal phenotype contribute to estrogen receptor expression within neural circuits involved in sleep and circadian regulation. Furthermore, the differential effects observed between the SCN and VLPO suggest that estrogen receptor regulation is region specific and highly dependent on the hormonal environment.

Interpretation

Sex differences in sleep regulation are thought to arise through interactions between gonadal hormones, sex chromosome complement, and neural circuitry involved in circadian and homeostatic regulation. The present findings provide evidence that estrogen receptor expression within the SCN and VLPO is differentially regulated by these factors and may contribute to previously observed sex differences in sleep behavior.

The SCN serves as the master circadian pacemaker and is responsible for coordinating daily physiological rhythms. Previous studies have demonstrated that estrogen receptor signaling within the SCN contributes to circadian regulation and sex differences in circadian phenotypes. The present findings are consistent with this literature, as significant differences in ER α expression were detected within the SCN in both genotype and phenotype comparisons under intact conditions. The observation that ER α , but not ER β , differed between groups suggests that ER α signaling may play a more prominent role in mediating sex-dependent circadian mechanisms.

The reversal in genotype-dependent ER α expression following gonadectomy further suggests that gonadal hormones interact with sex chromosome complement to regulate receptor expression. These findings are consistent with previous evidence demonstrating that organizational effects of pubertal hormones shape neural circuitry involved in sex-specific physiological regulation. Removal of gonadal hormones may therefore alter compensatory or regulatory mechanisms governing estrogen receptor transcription within the SCN.

In contrast to the SCN, few significant differences were observed within the VLPO under intact conditions. The VLPO is critically involved in sleep initiation and maintenance and has previously been implicated in sex differences in sleep homeostasis. Although receptor expression did not significantly differ between intact groups, gonadectomized phenotypic males exhibited significantly elevated ER α and ER β expression within the VLPO compared to females. These findings suggest that pubertal gonadal hormones may suppress or mask sex-dependent receptor expression differences within sleep-promoting regions.

Importantly, these data align with previous work demonstrating that gonadal hormones strongly influence sleep homeostasis and recovery sleep responses. While circulating hormones clearly contribute to sleep phenotypes, the persistence of receptor differences following gonadectomy further supports the idea that chromosomal sex and developmental hormone exposure independently shape neural systems involved in sleep regulation.

Additionally, gonadectomized XX females exhibited higher ER α expression within the VLPO compared to their intact counterparts, suggesting that circulating gonadal hormones may exert organizational or regulatory effects on estrogen receptor expression within sleep-promoting brain regions. These findings support the possibility that developmental hormone exposure contributes to long-term modulation of receptor signaling within the hypothalamus.

Phenotypic sex differences were also evident within the VLPO following gonadectomy. Phenotypic females demonstrated markedly greater ER α and ER β expression within the VLPO compared to phenotypic females following gonadectomy, suggesting that removal of gonadal hormones unmasks sex-dependent receptor differences within sleep-promoting regions.

Because the VLPO plays a central role in sleep homeostasis and sleep initiation, these distinct receptor expression patterns may provide a molecular basis for sex differences observed in sleep disorders such as insomnia. Fluctuations in gonadal hormone levels across puberty, reproductive aging, and stress exposure may therefore contribute to sex-specific vulnerability to sleep disruption. Together, these findings highlight the importance of considering both chromosomal and hormonal influences when developing targeted therapeutic approaches for sleep and circadian disorders.

Together, the present findings suggest that estrogen receptor signaling within the hypothalamus may represent one mechanism through which biological sex influences sleep and circadian physiology. The region-specific effects observed in the SCN and VLPO further highlight the complexity of sex-dependent neural regulation and emphasize the importance of examining both hormonal and genetic contributions simultaneously.

Limitations

Several limitations should be considered when interpreting the findings of the present study. One important limitation is sample size. Although significant differences were detected in several comparisons, variability within groups was relatively high, particularly in gonadectomized animals. Increasing sample size would improve statistical power and may reveal additional genotype- or phenotype-dependent effects that were not detectable in the present experiments.

Another limitation is that receptor expression was assessed only at a single circadian time point (ZT6). Estrogen receptor expression within the hypothalamus may fluctuate across the circadian cycle, and it is therefore possible that additional sex differences emerge at other time points. Future experiments incorporating multiple circadian sampling periods would provide a more comprehensive understanding of temporal regulation of receptor expression.

Additionally, the present study quantified receptor puncta counts but did not directly assess receptor functionality or downstream signaling pathways. Although fluorescent in situ hybridization provides sensitive spatial localization of ER α and ER β mRNA, changes in transcript abundance do not necessarily reflect alterations in protein levels or receptor activity. Future work incorporating immunohistochemistry, electrophysiological recordings, or transcriptional profiling would help clarify the functional consequences of altered receptor expression.

The present experiments also focused exclusively on the SCN and VLPO. While these regions are critically involved in circadian and sleep regulation, sleep behavior is mediated through broader neural networks including the basal forebrain, lateral hypothalamus, and brainstem arousal systems. It is likely that additional sex-dependent receptor differences exist within these regions as well.

Finally, although the FCG model allows separation of chromosomal and gonadal sex, developmental and environmental influences cannot be fully isolated. Organizational hormone effects occurring during early development may continue to shape receptor expression patterns into adulthood, making it difficult to fully dissociate genetic and hormonal contributions.

Future Directions

Future studies should expand upon the present findings by increasing cohort size and incorporating additional experimental conditions to further clarify the mechanisms underlying sex differences in sleep regulation. In particular, examining receptor expression across multiple circadian time points would help determine whether sex-dependent differences in ER α and ER β signaling exhibit temporal variation within the SCN and VLPO.

It would also be valuable to directly correlate receptor expression patterns with behavioral measures of sleep architecture and sleep homeostasis. Combining receptor quantification with EEG/EMG recordings following sleep deprivation or stress exposure would provide insight into how estrogen receptor signaling contributes to functional sleep outcomes.

Because the present study focused on baseline receptor expression, future experiments should also investigate how ER α and ER β expression change following acute sleep deprivation or psychological stress. Previous studies have demonstrated that stress and sleep loss differentially affect males and females, and the FCG model provides a unique opportunity to dissociate the contributions of chromosomal and hormonal sex to these responses.

Further investigation into the downstream molecular pathways regulated by estrogen receptor signaling may also provide mechanistic insight into sex-specific sleep phenotypes. For example, assessing immediate early gene expression, synaptic plasticity markers, or neurotransmitter systems downstream of estrogen receptor activation may clarify how receptor differences influence neural activity within sleep regulatory circuits.

Finally, these findings may have broader translational implications for understanding sex differences in human sleep disorders. Women exhibit higher rates of insomnia and stress-related sleep disturbances, yet the biological mechanisms underlying these differences remain poorly understood. Identifying how estrogen receptor signaling contributes to sleep regulation may ultimately improve development of sex-specific therapeutic strategies for sleep and circadian disorders.

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