PHOTIC AND TRANSCRIPTIONAL REGULATION OF THE REPRODUCTIVE AXIS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Neuroscience – Doctor of Philosophy

2025

ABSTRACT

Shift work is detrimental to many facets of female reproductive health, extending beyond just fertility but also affecting menstrual health, reproductive hormones, and pregnancy. To better understand how the circadian disruption that occurs during shift work impacts reproductive health, we must first understand the regulation of the suprachiasmatic nucleus (SCN) of the hypothalamus, which is the pacemaker and synchronizer of circadian rhythms. The goal of this dissertation is to determine how the regulation of the SCN by homeodomain transcription factors or mistimed light exposure impacts circadian rhythms, female fertility, and estrous cycles in a mouse model. First, we explored the post-developmental roles of the homeodomain transcription factors SIX3 and SIX6 within neuromedin-S–expressing neurons of the SCN. Conditional deletion of *Six6* had no measurable effect on circadian or reproductive function, while deletion of *Six3* resulted in shortened circadian behavioral rhythms and impaired sperm motility, suggesting distinct and non-redundant roles for these transcription factors in maintaining adult SCN function and reproductive output. Building on the importance of transcriptional regulation in SCN neurons, we next investigated the role of the SCN-enriched transcription factor VAX1 in Vasoactive Intestinal Peptide (VIP)-expressing neurons, which are critical for synchronizing circadian output to downstream systems. Female mice lacking *Vax1* in SCN VIP neurons exhibited weakened SCN rhythms, disrupted estrous cyclicity, reduced estrogen, and increased depressive-like behavior. Finally, to model an environmental form of circadian disruption, we used a rotating light (RL) paradigm that mimics human shift work. While some mice maintained regular cycles (RL-C), others became acyclic (RL-A), despite identical light exposure. Compared to control lighting and RL-C mice, RL-A mice had a reduction in circulating progesterone, a blunted luteinizing hormone response to exogenous gonadotropin releasing hormone, and fewer maturing follicles in the ovary. Interestingly, while both RL-C and RL-A groups had comparable conception rates to controls, exposure to RL resulted in reduced litter sizes, suggesting gestational risks independent of estrous cyclicity. Together, these findings provide insight into how circadian disruption, whether genetic or light-induced, may contribute to irregular reproductive cycles, hormone imbalance, and poor reproductive outcomes in women.

This dissertation is dedicated to Gary Douma.
I never stopped trying to solve the world's problems.

ACKNOWLEDGEMENTS

First, thank you to my mentor and advisor, Dr. Hanne Hoffmann. Thank you for seeing something in that college sophomore and giving her a chance. Thank you for pushing me to be the best I can be. Thank you to Duong Nguyen, Kierra Jursch, Krystal Jang, and Dr. Thu Duong. A special thank you to Dr. Alexandra Yaw for being an amazing co-worker, research partner-in-crime, and a very dear friend. Thank you for introducing me to the place you love and letting me borrow your dream. Thank you to my committee, Drs. Alexa Veenema, Gina Leinninger, and Lily Yan. You have each supported me in pivotal ways, and I will always remember that. Thank you to my funding sources, the National Institutes of Health, Eunice Kennedy Shriver National Institute of Child Health and Human Development (T32HD087166), and the National Institute of Health. Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship (1F31HD114529-01A1). Thank you to the Neuroscience Program, especially Dr. AJ Robison and the wonderful Eleri Thomas. To the rest of the 3rd floor of ISTB, thank you for encouragement, lunches, snacks, wine nights, crafts, pink Wednesdays, and grand ol times.

Thank you to my friends in the Bailey Scholar's Program. Sarah, Dustin, Marcie, Brendon, Mike, Luke, and Graham, you have all made this past year a joy. And to each and every Bailey Scholar: a massive thank you for letting me be a part of your learning journey.

Thank you to many dear friends. Jessica, Veronica, Abi, Brandon, Erynn, Hannah, Bonnie, Ethan, Bethany, Shannon, Avery, and Kyrene, I could not have done this without you.

Thank you, Van Loh/Telloyan family, for welcoming me with open arms and curious minds. Thank you, April, Andrew, Nathan, and Emma, for the many phone calls, chats of all lengths, jokes, and encouragement. Thank you, Tate, Xander, and Wesley, for being the cutest nephews ever born. Thank you to my parents, Doug and Carrie DeVries, for never doubting me, for helping me navigate the foreign territory of college, and for having my back through it all.

And finally, Isaac. You are my home, my heart, my love. Thank you for everything, I love you.

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CHAPTER 1: INTRODUCTION

Female reproductive health is a rapidly expanding field that includes the study of menstrual cycles, reproductive hormones, fertility, pregnancy, and menopause. In recent years, the importance of studying women's health has been emerging, as studies based only on male subjects have resulted in mistreatment and even death of female patients [1]–[5]. One of the many current threats to female reproductive health is the high prevalence of shift work in the modern world. Shift work, defined as working outside the hours of 9:00 am to 5:00 pm, has been shown to have negative impacts on many aspects of female reproductive health, including irregular menstrual cycles, increased menstrual discomfort, decreased fertility, and an increased risk for miscarriage [6] and is an indicated risk factor by the International Agency for Research on Cancer for breast cancer [7]. One contributing factor to these shift work-induced impacts is the disruption of circadian rhythms that occur due to the mistimed light exposure experienced in shift work. Circadian rhythms are behavioral, physiological, and cellular processes in the body that repeat with a rhythmicity of ~24 h. These processes include sleep/wake cycles [8]–[11], hormone release [12]–[14], cell activity [15], [16], metabolic activity [17]–[21], reproduction [22]–[25] and a wide variety of other processes. Circadian rhythms can be disrupted by a number of things, including mistimed light exposure, irregular hormones, and altered transcription factor expression, in turn impacting the downstream processes like female reproduction. The goal of this dissertation is to examine the impacts of transcription factors and light exposure as regulators of circadian rhythms and the downstream impact on reproductive hormone cycles and fertility. This work will begin to explore the mechanisms behind the negative impacts of circadian disruption, primarily focusing on female reproductive health.

The molecular clock and circadian rhythms

Circadian rhythms are driven by a transcriptional-translational negative feedback loop at the cellular level [26]. The core of this loop, known as the molecular clock, is comprised of the genes Circadian Locomotor Output Cycles protein Kaput (CLOCK) and Brain and Muscle Arnt-like protein 1 (BMAL1), which heterodimerize and bind to the DNA to regulate the transcription of Period (Per1/2/3) and

Cryptochrome (Cry1/2) genes, as well as a variety of other genes referred to as clock-controlled genes [27], [28]. PER and CRY in turn heterodimerize and inhibit the action of the CLOCK/BMAL1 complex, inhibiting gene transcription. The degradation of the PER/CRY complex then allows this process to begin again [29]. The entire feedback loop takes approximately 24 hours, driving the 24-hour rhythms on the cellular level. Through this transcriptional loop, the molecular clock drives expression of clock-controlled genes on the cellular level, generating 24-hour rhythms in cell function, ultimately leading to tissues and physiological processes aligned to the time of the day (Fig. 1.1) [30].

Circadian rhythms can be entrained (aka adjusted) to various stimuli. This allows the bodies internal rhythms to be aligned with the external environment. These stimuli are called Zeitgebers, a German term that translates to “time givers”. Zeitgebers are often divided into two categories: non-photic and photic [31], [32]. Non-photic cues include food [33]–[35], social interaction [36], [37], and exercise [38]–[40]. Photic cues, or light cues, are powerful, leading to prompt entrainment of circadian rhythms throughout the body [41], [42]. All of these cues need to be integrated and communicated to influence circadian rhythms. For this to occur, one localized area of the body primarily responsible for coordinating circadian rhythms and entraining to zeitgebers is the suprachiasmatic nucleus (SCN) of the hypothalamus (Fig. 1.1).

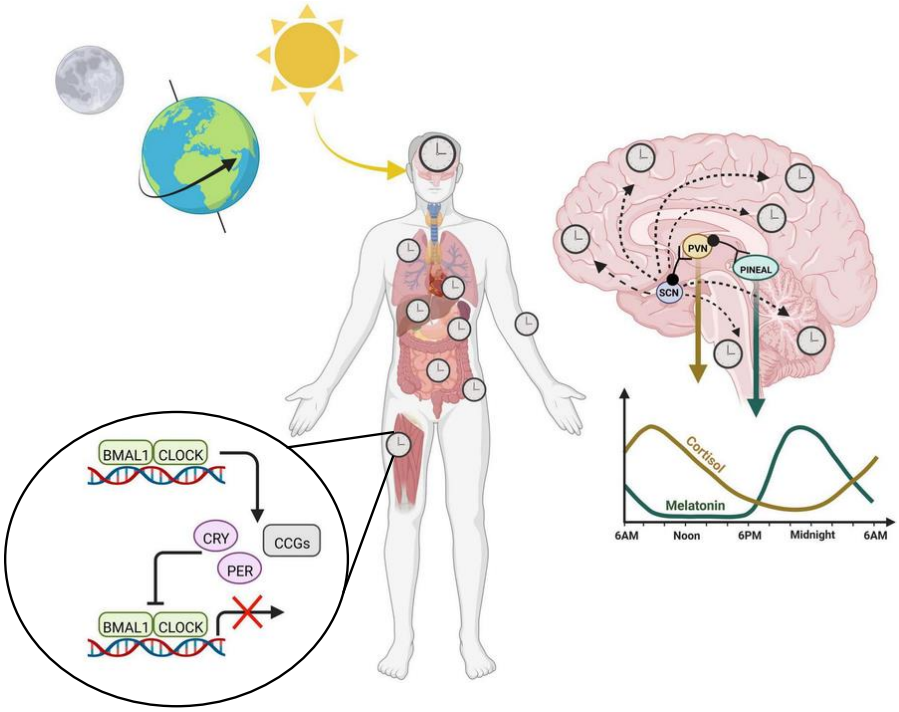

Figure 1.1. Circadian rhythms across the body are entrained by the 24-h environment. Diurnal light exposure entrains the "master clock", known as the suprachiasmatic nucleus (SCN). The SCN coordinates daily rhythms in the brain as well as the periphery via a combination of neuronal signals and hormones, including cortisol and melatonin, which fluctuate opposite to each other and promote sleep/wake cycles. At the cellular level, the core transcriptional and translational feedback loop in the nucleus is characterized by BMAL1 and CLOCK binding to E-box containing genes to regulate transcription of numerous clock-controlled genes (CCGs), including their own repressors, PERIOD (PER) and CRYPTOCHROME (CRY). Heterodimers PER and CRY translocate back into the nucleus to inhibit the CLOCK:BMAL1 complex, thereby inhibiting their own production. Used and adapted with permission from [43].

Transgenic models to manipulate circadian rhythms and the molecular clock

To study the molecular components of circadian rhythms, transgenic mouse models have proven to be valuable tools. These models include whole-body knockouts of clock genes such as *Bmal1* [44] and models with mutations to clock genes to avoid the compensation of other molecules such as the *Clock-Δ19* model [45]. To measure rhythms within specific cells or tissues, Period2::luciferase mice [46] are often used. The Period2::luciferase model was created using a knock-in to insert the gene for luciferase following the sequence for *Per2*, resulting in the transcription of destabilized luciferase each time *Per2* is transcribed. This method allows the use of equipment such as a Lumicycle, which quantifies the luminescence as a measure of PER2 expression, to measure tissue-level rhythms in nearly any tissues of the body [46].

While full body knock out models are beneficial to understand the role of a gene in the entire body, they are limited in their ability to determine the role of a gene in specific tissues or cell types. To target specific tissues and cell-types, the Cre-*LoxP* system is often used, allowing for conditional deletion of specific genes. In these models, *LoxP* sites are located on either side of the target gene in one mouse line. Another mouse line expresses Cre-recombinase in specific cell populations using promotors specific to a cell population or tissue type. When these mouse lines are bred, their offspring experience recombination in the cells where both Cre-recombinase and *LoxP* sites are present, excising the target gene [47]. Previous work, including from the Hoffmann Lab, which I was involved in, shows that there are also limits to these models, as Cre-recombinase can occasionally produce a phenotype [48] and may exhibit non-specific expression [49], though when these limits are properly accounted for using controls, conditional knock-out models are valuable tools in scientific research.

Light exposure and the SCN

Light is the strongest entrainer of the SCN. Non-vision forming cells from the retina, termed intrinsically photosensitive retinal ganglion cells (ipRGCs), project from the retina through the optic nerve to select brain regions, including the SCN [50], [51]. The light information communicated from the ipRGCs is translated by the SCN, which then projects to downstream brain regions to communicate the time-of-day information, allowing the brain and eventually the body to entrain to the photic cues [52]. This process

occurs through the ipRGC's glutamatergic release and following activation of the *Per* genes in the SCN neurons. Exposure to light results in a release of the peptides PACAP and glutamate from ipRGCs cells that innervate the SCN, which in turn leads to the phosphorylation of the transcription factor CREB, which enters the nucleus and binds to the promotor region to transcribe *Per1*, *Per2*, and *cFos* [30]. The change in *Per1* and *Per2* expression in turn, regulates the timing of the rest of the molecular clock, adjusting the timing of the SCN to match the environmental cue of light.

Through this entrainment process, mistimed exposure to light can disrupt circadian rhythms. A single exposure to mistimed light or a single time change, such as traveling to another time zone, may result in jet lag [53]. However, chronic exposure to mistimed lights or consistent shifting between schedules may result in shiftwork disorder, a condition based on the disruption of circadian rhythms with symptoms that include insomnia and exhaustion [54]. In addition, disrupted circadian rhythms have been shown to increase the risk of developing or worsening mood disorders such as depression and anxiety [55].

Circadian desynchrony and female reproduction

In humans, a common method of mistimed exposure is shift work, which increases the risk for irregular menstrual cycles, menstrual cycle pain, and infertility in women [6]. Rodent models have helped us separate the effects of mistimed lights from confounding factors, such as job stress. When chronically exposed to light shifts – 10 hour shifts every 3-4 days for 9 months – one group found that around half of the C57B/6 mice developed irregular estrous cycles [56]. Others have found that mistiming of signals within specific organs of the HPG axis can impair fertility, such as irregular timekeeping in the ovary [57], [58]. While these studies all point to mistimed light exposure resulting in impaired female reproductive health, the mechanisms and level of mistiming of tissues throughout the HPG axis have yet to be explored.

Circadian desynchrony and male reproduction

The impacts of circadian dysregulation in male subjects is still debated. Most studies suggest that circadian timing is less crucial to male reproduction compared to female reproduction [25], with a few studies indicating that shift work and other causes of mistimed light can cause a minor impairment to male reproduction [59], [60]. One such study found that men working in night or rotating shifts had a small, but

significant decrease in sperm counts, as did a mouse model of shift work [59]. However, unpublished studies of the Hoffmann lab, did not identify a change in sperm production or motility using a shift-work model (unpublished). The exact mechanism of these changes is unknown and many reviews agree that more research is required to determine the severity of any impacts shift work may have on male reproduction [61], [62].

The Suprachiasmatic Nucleus and the Reproductive axis

Circadin rhythms and the SCN are important at every stage of the reproductive axis, also known as the hypothalamic-pituitary-gonadal (HPG) axis. Circadian rhythms are coordinated by the SCN, located within the hypothalamus of the brain [63], [64]. Disruption of the SCN results in fertility issues within females. In rodent studies, ablation of the SCN, as well as treatment with tetrodotoxin (TTX) via a cannula to inhibit the neuronal function of the SCN, results in a failure to ovulate [23], [65]. Interestingly, while ablation of the SCN prevents ovulation, fetal transplants of SCN tissue to the third ventricle of mice with an ablated SCN do not restore the signaling required for the LH surge, indicating that this signaling is driven by neuronal connections and cannot be restored through diffusion like locomotor rhythms, which are restored with a transplant [66]. This supports the idea that direct neural connections from the SCN to downstream brain regions are required for ovulation and female reproduction. Recent work has explored the cellular makeup of the SCN and the role of the diverse populations of neurons found within the structure [67]. These neuron populations play different roles in circadian regulation, and are often defined by the neuropeptides they produce [64], [68], [69]. The diverse populations of neurons within the SCN are required for different functions both within the SCN and to communicate with surrounding brain regions. The neurons in the SCN express a variety of neuropeptides [63], [70]–[72], including Gastrin-Releasing Peptide (GRP), vasoactive intestinal peptide (VIP), vasopressin (AVP), and neuromedin-S (NMS), where the focus here will be on VIP, AVP, and NMS (Fig. 1.2). These neuropeptides, as well as their function in the regulation of circadian rhythms and reproduction are outlined below.

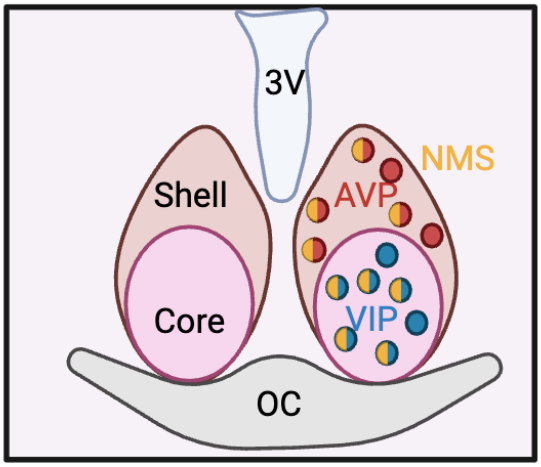

Figure 1.2. The structure and key neuronal populations of the suprachiasmatic nucleus (SCN) studied in this project. The SCN is a bilateral structure composed of a shell and a core. The core is located directly above the optic chiasm (OC) and the nuclei are on either side of the 3rd ventricle (3V) within the base of the hypothalamus. The neuron populations studied include vasopressin (AVP), which is located primarily in the shell, vasoactive intestinal peptide (VIP), located primarily in the core, and neuromedin-S (NMS), which is co-expressed with a large portion of both AVP and VIP neurons throughout the SCN. Figure made with Biorender.com.

SCN VIP neurons in circadian regulation

Within the core of the SCN, 10% of the neurons express VIP [70], an important peptide in entrainment and circadian rhythms coordination. VIP neurons are known to be involved in the reception of light information from the optic nerve, which is located directly below the SCN (Fig. 1.2), and using that light information to entrain the SCN to the environmental light patterns [73], [74]. To do this, VIP neurons project to other neurons, including AVP-expressing neurons, to synchronize the SCN [70], [75]. VIP-expressing neurons from the SCN have been shown to play important roles in regulating the length of circadian rhythms, known as the period. Specifically, loss of VIP peptide from the SCN results in shortened rhythms [76]–[78], indicating that neurons within the SCN that express VIP are involved in maintaining the endogenous 24-hour rhythm. VIP neurons within the SCN project to a variety of other brain regions to synchronize them to zeitgebers, such as the paraventricular nucleus of the thalamus where VIP suppresses glucocorticoid release [79]–[81], the dorsomedial hypothalamic nucleus which is involved in locomotor rhythms [78], and the medial preoptic area where VIP neurons activate GnRH neurons [52], [82], [83], the latter which is a required hormone for reproductive function.

SCN VIP neurons in female reproduction

VIP neurons from the core of the SCN project both directly and indirectly to gonadotropin-releasing (GnRH) neurons in the preoptic area of the brain [84]. Indirect signals from VIP neurons project first to AVP neurons within the shell of the SCN which then project to kisspeptin neurons in the anteroventral paraventricular nucleus, that in turn project to the GnRH neurons, which regulate hormonal release from the pituitary gland. GnRH neurons signal the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) which maintain ovarian health and follicle development in females [85] and regulate testicular growth and sperm production in males (Fig. 1.3) [86]. Due to this role in the HPG axis, it comes as no surprise that VIP-expressing neurons of the SCN are important in female reproduction. One study shows that full body VIP knock out mice have lengthened estrous cycles and a reduction in oocytes [77]. This study was limited due to the nature of the global knock out. Another study that selectively ablated VIP neurons within the SCN similarly found lengthened estrous cycles and found similar results if

VIP receptors were deleted from GnRH neurons [87]. Surprisingly in both cases, the mice still ovulated, though its frequency was decreased. This suggests either a compensatory mechanism that has not yet been identified or supports the idea that VIP plays a modulator role of the reproductive axis and is not alone responsible for the prompting of ovulation.

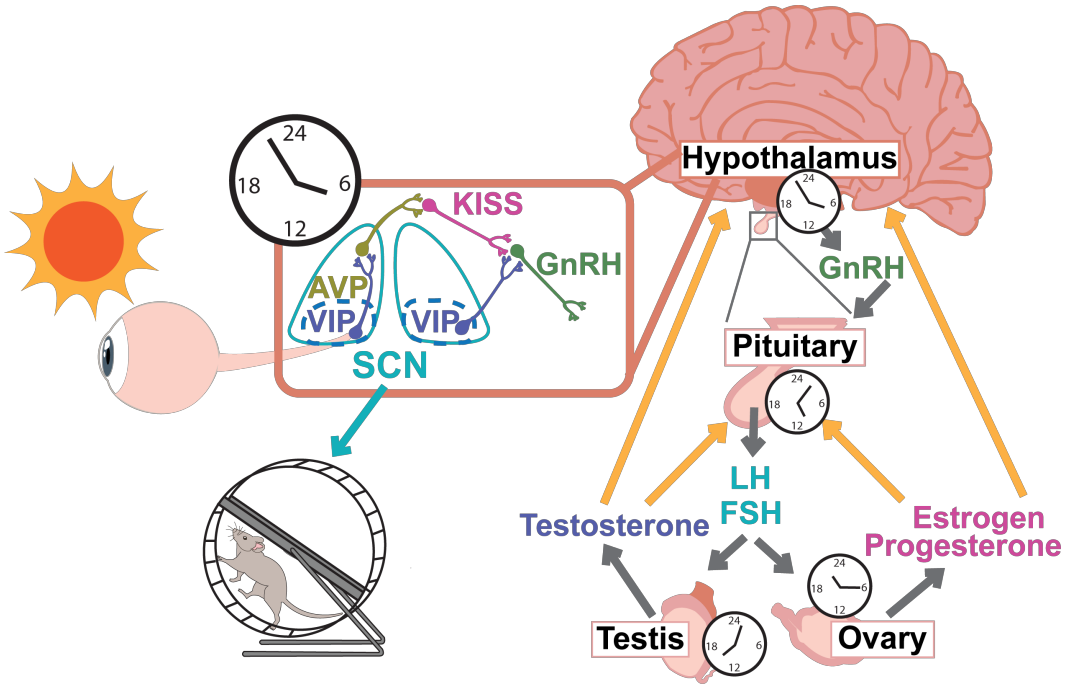

Figure 1.3. The hypothalamic-pituitary-gonadal axis is regulated by the suprachiasmatic nucleus of the hypothalamus. SCN = Suprachiasmatic nucleus. VIP = vasoactive intestinal peptide. AVP = Vasopressin. KISS = Kisspeptin. GnRH = Gonadotropin Releasing Hormone. LH = Luteinizing Hormone. FSH = Follicle Stimulating Hormone.

SCN AVP neurons in circadian regulation

Twenty percent of the neurons within the SCN express AVP, a majority of which are located in the shell (Fig. 1.2) [70]. AVP neurons receive neural projections from VIP neurons in the core and are known to be important in communicating rhythms and intra-SCN coordination, though the exact mechanism is unknown [70], [88]. AVP neurons in the SCN are known as weak synchronizers, playing a less prominent role in the synchronization of the brain than VIP neurons, however, they still function to synchronize the SCN, functioning in a compensatory manner when VIP or VIP receptors are absent [89], [90]. Research into the roles of AVP has proven more challenging than VIP, as AVP knockout mice do not survive past postnatal day 7 [91]. The neurons that express AVP, however, have been studied using the conditional deletion of important clock genes. The loss of *Bmal1* from AVP neurons results in lengthened rhythms and a few mice developed arrhythmic behavior [90]. To communicate time of day information as well as other signals, AVP neurons project to various regions outside of the SCN, including the arcuate nucleus and the median preoptic nucleus to control the timing of body temperature [92], the paraventricular nucleus to regulate endocrine and autonomic rhythms [93], [94], and to the organum vasculosum of the lamina terminalis to regulate thirst and drinking behavior [52], [95].

SCN AVP neurons in female reproduction

AVP neurons also regulate GnRH neurons indirectly through their projections to Kisspeptin neurons in the arcuate nucleus and the anteroventral periventricular nucleus (Fig. 1.3) [96], suggesting a role for them in ovulation and reproduction as well. However, to date little work has examined their exact role in female reproduction aside from their activation of kisspeptin. One study found that treating the SCN with an AVP antagonist via intracerebroventricular injection results in a reduced LH surge [97], however it did not entirely eliminate ovulation. Another study found that loss of *Bmal1* from AVP neurons had no effect on female reproductive health [98]. More work will be required to examine the exact function of SCN AVP neurons in relation to reproduction.

SCN NMS neurons in circadian regulation

Neurons that express AVP or VIP have also been found to coexpress NMS, a relatively understudied neurotransmitter in the SCN (Fig. 1.2) [67], [78]. NMS is the most recently discovered (2005) as well as the most abundantly expressed neuropeptide in the SCN, as it is expressed in 40% of SCN neurons [99]. While the role of NMS in the SCN is not yet clear, these neurons have been found to be important in regulating circadian rhythms. Silencing these neurons using a tetO-tetanus toxin (TeNT) mouse line crossed with an NMS-cre mouse line results in lost locomotor rhythms and disrupting the molecular clock in NMS neurons through the loss of *Bmal1* or the overexpression of *Per2* results in dysregulated in vivo rhythms [100]. These effects may be due to the silencing of neurons that co-express VIP/NMS or AVP/NMS, inhibiting the release of VIP or AVP, resulting in similar circadian phenotypes to *Vip* or *Avp* knock-outs, though this remains undetermined. Current studies are still emerging discovering the projections of SCN NMS neurons, with recent work finding projections to the dopaminergic neurons in the paraventricular nucleus, providing a mechanism by which these neurons can communicate seasonal changes in light information to the body [101].

SCN NMS neurons in female reproduction

As the most recently discovered neuropeptide in the SCN, very little has been explored in relation the role of NMS neurons in female reproduction. One study suggests that, in female rats, NMS may regulate LH expression, a pituitary hormone required for reproductive function, though the mechanism is unexplored [102], but not much else is known to date.

The effects of circadian disruption on reproductive health

Each portion of the HPG axis must be in synchrony for reproduction to occur [103], [104]. The hypothalamus, pituitary, and gonads function in a circadian manner and their synchrony is required for successful reproduction [105]. During non-ovulatory phases of the menstrual cycle, the SCN coordinates the rhythms of the HPG axis, in turn promoting the release of the steroid sex hormones estrogen and progesterone, which change across the menstrual cycle (Fig 1.4) and are important for female reproductive health [106], [107]. In addition to its role in regulating hormone release, the SCN is important for the timing

of ovulation [57], [108]. In the presence of a threshold estrogen level, GnRH neurons, when activated by SCN VIP neurons, send a high-amplitude signal to neurons within the pituitary gland to release a large quantity of LH, known as the LH surge, resulting in ovulation in females [109]. The SCN regulates this surge, resulting in ovulation following a circadian pattern, with the peak around activity onset. Therefore, disruption of circadian rhythms often impacts fertility and ovulation.

The reproductive axis can be disrupted through frequent exposure to mistimed light. Light at night has been shown to alter the length of menstrual cycles in humans [110]. In rodents, exposure to constant light disrupts ovulation in hamsters [111] and completely inhibits ovulation through LH suppression in rats [112]. As the SCN is comprised of neurons, and neuronal signals are relatively quick to adjust to input, the SCN can adjust to irregular exposure to light within minutes, depending on the magnitude of the adjustment required [113]. However, the rest of the body, including the reproductive axis, takes longer to adjust as it relies primarily on hormonal signaling to convey the time-of-day information [13], [114]. This results in misalignment between the SCN and downstream tissues [115], [116], which is likely to impair fertility as each stage of the axis relies heavily on circadian time keeping.

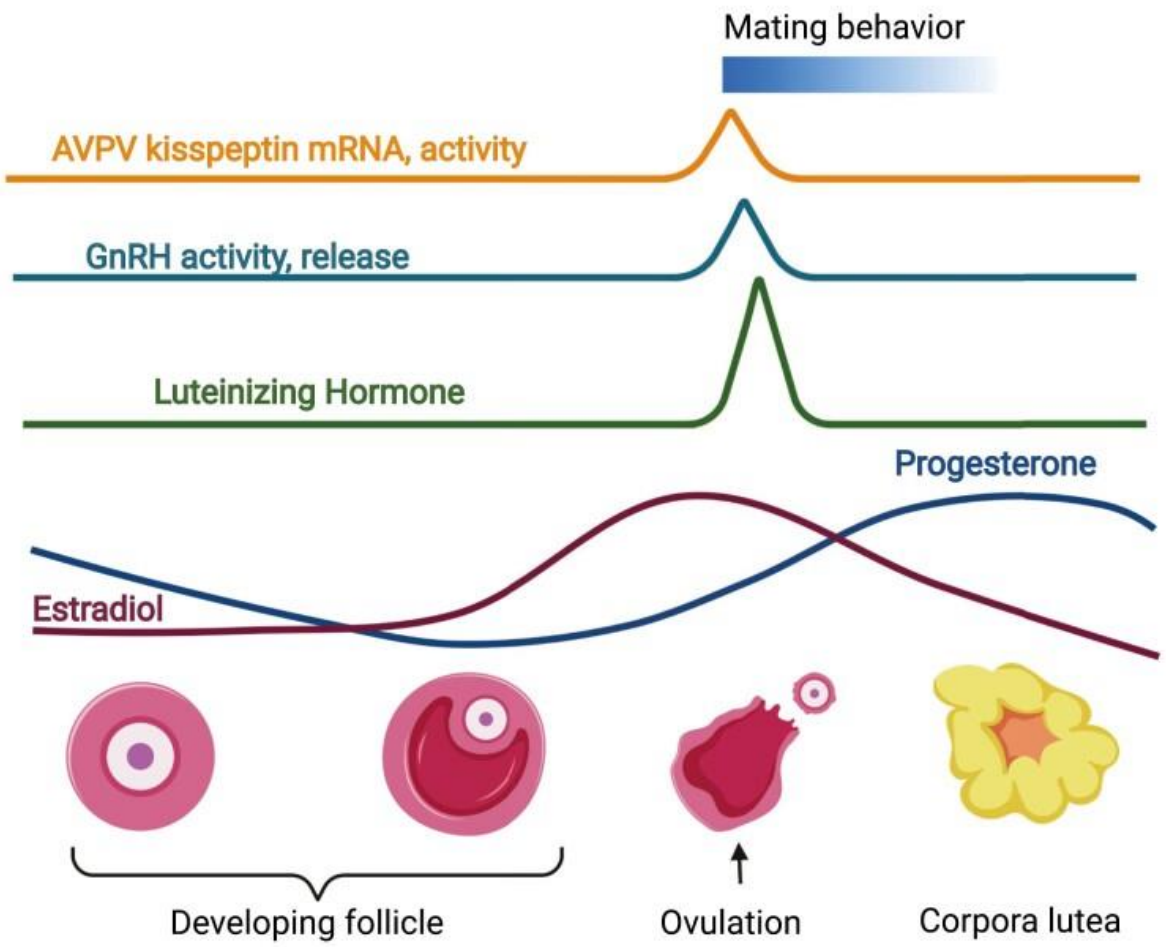

Figure 1.4. Ovulation is precisely timed to increase the chances of fertilization of the oocyte during its short life span. In most spontaneous ovulating mammals, the timing of ovulation happens shortly after activity onset/wake-up in both diurnal and nocturnal species. This timing of ovulation is thought to increase the chances of fertilization of the oocyte, which only lives 12 to 24 hours. Ovulating at activity onset increases the chances of mating behavior, which occurs during the wake hours. A sophisticated neuroendocrine-ovarian feedback mechanism generates increased estradiol levels, which are required for the LH (luteinizing hormone) surge, which promotes ovulation and female sexual behavior. Estradiol is produced by the maturing follicle, whereas the increase in progesterone is driven by the corpus luteum in the ovary that forms after ovulation. Progesterone is a primary hormone that prepares the uterus for implantation and successful pregnancy maintenance. Used with permission from [14].

Homeodomain transcription factor expression in the SCN and their importance in SCN neuronal function

Transcriptional regulation is important to the function and survival of cells, including the neurons of the SCN, giving cells the ability to alter function and respond to both intra- and extra-cellular stimuli [117]. Transcription can be regulated through a variety of processes, including DNA methylation, promotors, and transcription factors. Transcription factors are proteins that regulate the transcription of genes by recruiting proteins such as RNA polymerase to promote transcription or by suppressing transcription [118]. Transcriptional regulation is required for the transcriptional-translational loop of the molecular clock (described above) and plays roles in many aspects of circadian regulation.

Transcription factors enriched in the SCN

A unique feature of the SCN is the enrichment of transcription factors primarily known for their role in brain development, which maintain a high expression within the SCN throughout life. These SCN-enriched transcription factors share a helix-turn-helix DNA-binding motif known as the homeodomain [119]. Homeodomain transcription factors target TAAT sequences in the DNA and promote the transcription of downstream genes. To date, four such transcription factors have been identified; Ventral Anterior Homeobox 1 (VAX1), Sin Oculis 3 (SIX3), Sin Oculis 6 (SIX6) [120], [121] and Lim Homeobox 1 (LHX1, LHX1 is not studied in this dissertation). These four homeodomain transcription factors are expressed in the developing hypothalamus, specifically the region that gives rise to the SCN [67], [122]. Deletion of any of these transcription factors prior to the development of the SCN (which occurs around embryonic day 12.5 in the mouse [123]) results in a failure of SCN formation [120], [122], [124]. Interestingly, along with their role in SCN development, these transcription factors have been shown to remain present in the adult mouse SCN, though their roles in adulthood remain largely underexplored [67], [125]. Deletion of these transcription factors after SCN formation (around embryonic day 16) does not cause a loss of SCN morphology, but may impact SCN function, demonstrating the importance of these

transcription factors after development, a topic I will focus on in this dissertation. In Table 1.1 I have summarized what we know about SIX3, SIX6, and VAX1 regarding SCN function and reproduction.

Table 1.1. Current data on the role of homeodomain transcription factors in mouse models [121], [126].

Gene	Homozygous Knock Out	Heterozygous Knock Out	Synapsin-cre	Clock Genes Regulated	Neuro-peptides Regulated
Six3	Embryonic Lethal	Impaired female fertility, impaired male smell and mating behavior Normal SCN morphology	Reduced male and female locomotive rhythmicity Reduced female fertility Dwarfism and metabolic changes in male Normal SCN morphology	↑ Per2	↑ AVP --- VIP
Six6	No SCN Underdeveloped optic nerve	No reproductive phenotype	NA	Ch. 2	NA
Vax1	Lethal at birth	Impaired male and female reproduction Normal SCN morphology	Reduced locomotive rhythmicity Impaired female fertility Normal SCN morphology	Ch. 3	↑ VIP --- AVP

SIX3 and SIX6 regulate circadian output

The transcription factors SIX3 and SIX6 are closely related members of the *Six* family of genes known for their role in brain development and, specifically, eye development [127]. They are often thought to have similar or compensatory functions, though some work has shown that this may not always be the case [125]. In *in vitro* transfection studies, it has been found that SIX3 upregulates the expression of AVP as well as PER2 but has no effect on VIP expression [121], indicating its role in circadian regulation. Transfection studies have yet to be conducted examining the role of SIX6 in the regulation of circadian-related genes. Using in vivo models, homozygous loss of *Six3* results in a failure to develop the anterior portion of the head and brain, leading to fetal death [128], while homozygous loss of *Six6* results in underdeveloped optic nerves and no development of the SCN [129]. Previous work by the Hoffmann Lab used Synapsin-cre, which is expressed almost exclusively in mature neurons [130], to cause recombination of *Six3* in neuronal cells after embryonic day 12.5. This results in reduced locomotor rhythmicity in males and females [131]. While this suggests a role for *Six3* in the functions of the SCN, Synapsin-cre lacks the specificity to conclude that these deficits are due to loss of *Six3* in the SCN specifically. No work to date has examined the role of *Six6* in adult SCN function.

Six3 and Six6 regulate female reproduction

Due to their role in the development of the SCN, studies exploring the roles of *Six3* and *Six6* in reproduction are difficult and limited. Previous work from the Hoffmann lab has shown that the use of Synapsin-cre to cause recombination of *Six3* in mature neurons results in impaired female fertility as evidenced by a reduction in the number of litters produced in 4 months [121], [131]. Full body SIX6 knock-out mice also have reduced reproductive health with long estrous cycles and reduced ovarian weight [132]. Though, this effect is likely due to the role that SIX6 plays in GnRH neuron migration, as the knockout mice had a significant reduction in GnRH. While both of these studies suggest roles for SIX3 and SIX6 in female reproduction, neither are SCN specific and cannot determine the role that these transcription factors play in the SCN's regulation of reproduction.

VAX1 regulates circadian output

Previous work has shown that homozygous loss of *Vax1* results in holoprosencephaly and cleft palate, resulting in fetal death. Due to VAX1 knockout mice dying within a day of birth, to investigate its role in SCN neuron function, heterozygous and conditional knockout models have been used. The Hoffmann lab has shown that VAX1 is necessary for SCN development and function [120], [121], [124], and that heterozygous loss of *Vax1* results in impaired fertility in both male and female mice [126]. We have also found that selectively deleting *Vax1* from cells that express Synapsin-Cre, a cre driven by mature neurons results in reduced timekeeping output from the SCN [121]. VAX1 has also been shown to promote the transcription of VIP within the SCN [120], [121], suggesting that it may play a role in neuropeptide regulation in the adult SCN.

VAX1 regulates female reproduction

In addition to its role in regulating circadian rhythms, VAX1 has been shown to be involved in reproductive health. Synapsin-cre conditional knock outs have given some insight into the roles of VAX1 in reproduction. Dr. Hoffmann's previous work found that recombination of *Vax1* in mature neurons results in a mouse line with impaired fertility, specifically a reduction in litters in 4 months and a reduction in LH released in an induced surge [121]. These studies are limited by the lack of specificity in Synapsin-cre, leaving the role of VAX1 in the SCN in relation to reproductive health undetermined.

Mouse as a model

It is important to mention that throughout this dissertation, all experiments were conducted in mouse models. Mouse models are highly useful in modern science due to our understanding and ability to manipulate the mouse genome. These studies rely on conditionally genetically modified animals to answer questions, requiring a model such as the mouse. For this work specifically, a mouse model was also used because the SCN is a highly conserved structure in mammals [72], and in both diurnal and nocturnal mammals it is regulated by light [133], [134]. These benefits outweigh the limitation of using a nocturnal animal model to begin to explore potential mechanisms to benefit a diurnal species.

Research question

This dissertation seeks to answer how regulating the SCN by homeodomain transcription factors or by irregular light exposure impacts circadian rhythms, reproductive function, estrous cycles, and hormone release. I hypothesize that dysregulation of the SCN through loss of transcription factors *Six3*, *Six6*, or *Vax1* or mistimed light exposure, will negatively impact reproductive health in mice.

To answer this hypothesis, I have developed three independent chapters. Chapter 2 focuses on the contribution of *Six3* and *Six6* in NMS neuron function and SCN output in males and females (manuscript in preparation); Chapter 3 focuses on the role of *Vax1* in VIP neuron function, SCN output and female reproductive function (Published, Van Loh *et al.* 2023), and Chapter 4 uses a novel mouse model I have developed that mimics the irregular sleep-wake cycles of shift workers, to understand how such alternating light exposure impacts SCN function and reproduction in females (manuscript in preparation).

CHAPTER 2: THE TRANSCRIPTION FACTORS SIX3 AND SIX6 DIFFERENTIALLY AFFECT CIRCADIAN RHYTHMS IN NEUROMEDIN-S NEURONS

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Author Contributions: All authors provided contributions to study conception and design, acquisition of data or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published. Here are the most important contributions of each author: H.M.H., L.E.C., B.M.V.L., K.J.T., P.L.M., and M.R.G. designed the study. Data were collected by G.A.D., L.E.C., M.M.P, N.C.P.N., B.M.V.L., D.N., J.C., K.J.T., and H.M.H. Analysis was carried out by B.M.V.L., G.A.D., M.M.P., N.C.P.N., J.C., H.M.H., and K.J.T. The manuscript was written by B.M.V.L., G.A.D, K.J.T. and H.M.H. All authors approve and take final responsibility for this article.

Abstract

Circadian rhythms repeat approximately every 24 hours and in mammals are regulated by the suprachiasmatic nucleus (SCN) in the hypothalamus of the brain. The regulation of circadian rhythms and downstream processes is highly dependent on the proper development and function of the SCN. *Six3* and *Six6* are homeodomain transcription factors that are both required for SCN development; however, both *Six3* and *Six6* remain expressed in the adult SCN. While a nervous-system wide postnatal role of *Six3* has

been described, the actions of *Six3* and *Six6* in the adult SCN are largely unknown., To determine the role of *Six3* and *Six6* in the SCN after early development, we conditionally deleted either *Six3* or *Six6* from cells that express neuromedin-S (NMS), a neuropeptide expressed in the majority of neurons within the SCN, which are cells known to be involved in SCN circadian output and reproductive function. We found that the NMS-Cre allele turns on in the SCN after E16.5, which allows the investigation of the role of *Six3* or *Six6* in the SCN after the majority of SCN neurogenesis. We hypothesized that *Six3* and *Six6* in NMS neurons regulate SCN circadian output and resulting reproductive function in males and females. To that end, we analyzed reproductive and circadian function of *Six3* and *Six6* in the SCN using conditional deletion of each gene in NMS-Cre expressing neurons. We found that loss of *Six6* from NMS neurons had no impact on puberty and reproduction. However, loss of *Six3* from NMS neurons resulted in significantly decreased sperm motility, potentially through direct effects of *Six3* in the testis. We next examined the roles of *Six3* and *Six6* in locomotor rhythms. We found that loss of *Six3,* but not *Six6*, in NMS neurons resulted in shortened wheel-running periods in constant darkness, indicating a shortening of the rhythm within the SCN. Together, these data indicate differing roles of *Six3* and *Six6* in the adult SCN.

Introduction

Daily rhythms coordinate biological processes in the body that repeat approximately every 24 hours. In mammals, these circadian rhythms are synchronized by the hypothalamic brain region known as the suprachiasmatic nucleus (SCN). Without proper SCN function, rhythms may be irregular or absent, affecting downstream processes such as reproduction [22], [135], sleep [136], [137], and metabolism [18], [19], [138]. Many genes and transcription factors regulate the development and continued function of the SCN [122], [123].

The development of the SCN relies on transcription factors known as homeodomain transcription factors, including Six Homeobox 3 (*Six3*) and Six Homeobox 6 (*Six6*) [122], [125], [139]. Loss of either *Six3* or *Six6* during the developmental period negatively impacts SCN formation, circadian regulation, and reproduction. In mice, homozygous loss of *Six3* results in failure to develop the anterior portion of the head and brain, resulting in fetal death [128]. Previous work has shown that flox-mediated recombination of *Six3*

at embryonic day 12.5 [140] using a neural-specific driver, Synapsin-Cre, results in irregular circadian rhythms in both male [131] and female mice [121], as well as impaired fertility in female mice [121]. However, as Synapsin-Cre is expressed in most mature neurons as well as extra-CNS tissues, it is unclear which of these effects are due to the loss of *Six3* in the SCN specifically. Heterozygous loss of *Six3* is non-lethal, instead resulting in pituitary gland dysmorphology, impaired olfaction, and a reduction in male reproductive behavior [141], [142]. Homozygous loss of *Six6* leads to variable development of the optic nerves, optic chiasm, and SCN; behavioral rhythms are impaired or absent [129]. Interestingly, *Six3* and *Six6* expression is maintained almost exclusively in the SCN after development. However, the potential function of these homeodomain transcription factors in the SCN after development is unknown [67], [125]. We hypothesized that in addition to the known developmental role of *Six3* and *Six6,* these two transcription factors are also involved in post-developmental regulation of circadian function and downstream circadian processes. To test this hypothesis, we utilized a conditional knockout model wherein cre recombinase was expressed under the control of a Neuromedin-S (NMS) promoter, a neural peptide widely expressed in the SCN in the late-infantile and early juvenile stages [99], [100], [143]. This approach delayed the recombination of *Six3* or *Six6* until after SCN neurogenesis, which occurs between embryonic day 11-15 [122], allowing us to better investigate the roles of *Six3* and *Six6*..

Methods

Mice

All animal procedures were performed according to protocols approved by the University of California, San Diego Institutional Animal Care and Use Committee and the Institutional Animal Care and Use Committee of Michigan State University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Mice were maintained on a light/dark cycle of LD (12 h light, 12 h dark) with light parameters previously reported [143]. *Six6*$^{flox/flox}$ mice (RRID:MMRRC_068240-UCD) [139], were crossed with heterozygous NMS-Cre mice [RRID:IMSR_JAX:027205 ; JAX #027205] [100] to generate mice discussed here as *Six6*^{NMS}. Similarly, *Six3*$^{flox/flox}$ (RRID MGI:6507739) mice were crossed with NMS-Cre to generate *Six3*^{NMS} mice; VIP Cre

mice (RRID : IMSR_JAX:010908) to generate *Six3*^{VIP}; or AVP Cre mice to generate *Six3*^{AVP} mice [144]. In all conditional knockouts, the cre was maintained as heterozygous to avoid off-target effects of the Cre [48]. Cre negative, flox/flox littermates were used for controls. The Ai14 rosa tdTomato reporter mice (RRID:IMSR_JAX:007914**)** were crossed with NMS-Cre mice to create tdTomato$^{+/wt}$ NMS^{cre} (tdTomato^{NMS} mice) to visualize cre-containing neurons. Period2::Luciferase (PER2::LUC) mice were purchased from JAX (strain B6.129S6-Per2tm1Jt/J, JAX #006852; RRID:IMSR_JAX:006852) and crossed with the *Six6*^{NMS} mice and offspring heterozygous for PER2::LUC were used. Genotyping primer sequences were as follows: NMSiCre-wtF: CCGCATCTTCTTGTGCAGT, NMSiCre-wtR: ATCACGTCCTCCATCATCC, NMSiCre-creF: CCAAGTTAGCCTTCCATACACC, NMSiCre-creR: AGACGGCAATATGGTGGAAAAT, VIP Cre F: GCATTACCGGTCGTAGCAACGAGTG, VIP Cre R: GAACGCTAGAGCCTGTTTTGCACGTTC, Six6FL-WT/fl: GAAGCCCTTAACAAGAATGAGTCGG, Six6Flox-WT-loxp-R4: TCCCTTTGAATTTGGGTCCCTG, Six6Flox-R: CTTCGGAATAGGAACTTCGGT. Six3 F1: TGCCCCCTGCTAAAGAGCCAGT, Six3 R1: TAGGGACAGGCACGGAGGGTTG, Six3 R2: ATGCCCACATTGTCGGCCCATG; Rosa tdTomato F1: GGCATTAAAGCAGCGTATCC, Rosa tdTomato R1: CTGTTCCTGTACGGCATGG, Rosa tdTomato F2: CCGAAAATCTGTGGGAAGTC, Rosa tdTomato R2: AAGGGAGCTGCAGTGGAGTA. Mice were screened for germline recombination using the primer combinations listed above, and mice with germline recombination were excluded from the studies.

Cell Culture and transient transfections

A detailed protocol on cell culture and transient transfections has been previously described [131]. In brief, NIH3T3 (RRID:CVCL_0594) fibroblast cells were incubated in 10 cm plates in an incubator at 37°C with 5% CO_2 and maintained in complete media containing Dulbecco's Modified Eagles Media (DMEM), 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were transfected with a *Six6* expression vector [132] or empty vector (pcDNA) and a reporter vector: Rat *Avp*-luc (a gift from Robert Shapiro,Shapiro, Xu, and Dorsa 2000) or Pgl2 backbone; mouse *Vip*-luc [146] or Pgl3 backbone; mouse *Bmal1*-luc (a gift from Steven Brown (Addgene plasmid # 46824; http://n2t.net/addgene:46824 ;

RRID:Addgene_46824) [147] or Pgl; or mouse *Per2*-luc [a gift from Cheng Lee (Addgene plasmid # 16204 ; http://n2t.net/addgene:16204 ; RRID:Addgene_16204)] [148] or Sport2. To control for efficient transfection, reporter plasmid containing β-galactosidase constitutively driven by the Herpes virus thymidine kinase promoter (TK-βgal) was used.

On day one, cells were plated at 30,000 cells/well in a 12-well plate and incubated overnight to allow cell adherence. On day two, cells were transfected with the following amounts of plasmid: TK-βgal at 20 ng/well, luciferase plasmids or empty luciferase backbone at 400 ng/well, and pcDNA-empty vector (EV) or *Six6* expression vectors at 400 ng/well. Transfections were performed using the reagent Polyjet. On day three, 24 hours post-transfection the media was changed to fresh, complete media. On day four, 24 hours post media change, the cells were harvested in lysis buffer (100 mM potassium phosphate pH 7.8 and 0.2% Triton X-100) and plated in 96-well plates and read for luciferase and β-gal assays in a Luminometer microplate reader (Glomax, Promega). For the luciferase assay, luciferase assay buffer (dd H2O, 0.25 M Tris pH 7.8, 1 M MgSO4, 0.25 M ATP, and Luciferin). Within each well, luciferase values were normalized to TK-βgal values, then replicate luciferase/TK-βgal values were averaged. Luciferase was normalized to the luciferase backbone for both *Six6* and pcDNA. Fold change is displayed as normalized Six6/pcDNA.

Timing of NMS^{cre} activation

NMS iCre male mice were mated with tdTomato+/+ females and checked daily for vaginal plugs. On embryonic day 16, the dams were sacrificed and pups retrieved, decapitated, and the heads drop-fixed in 4% PFA. Tails were genotyped for Cre and SRY. After fixation, heads were sunk in 30% sucrose and ultimately embedded in O.C.T. (Tissue-Tek, Sakura) and stored at -80 C until sectioning. Heads were sectioned coronally at 20 μm in two series. One series underwent gentle washing in PBS for 20 minutes followed by incubation in 1 μg/mL DAPI for 6 minutes, followed by another 20-minute PBS wash. Slides were imaged at 20x using Zeiss Axio Imager M2.

Pubertal onset

Beginning at PND21, pubertal onset was established by visual inspection of preputial separation (PPS) in males and vaginal opening (VO) in females, as described previously [149]. Body weight was recorded daily until pubertal onset was observed.

Sperm count

Following euthanasia in males, testes, seminal vesicles, and epididymis were collected and weighed. Sperm was collected from epididymis of male mice in M2 media (Sigma #M7167). The epididymis was cut in half and sperm were expelled by gently pressing down on the epididymis and then left in M2 media at room temperature for 15 min. The numbers of total and motile sperm were counted from a 1:10 dilution of the M2 media containing sperm by using a hemocytometer. The second epididymis was cut into small pieces and left 15 minutes at room temperature in M2 media. The solution was homogenized frequently to help liberate the sperm. The solution was filtered using a cell streamer (70 μm, Falcon #352350) and sperm were diluted 1:10 with MQ before counting total number of sperm heads.

Plugging assay

To assess male plugging behavior, *Six3*$^{flox/flox}$ or *Six3*^{NMS} male mice were paired with a virgin WT female for 10 days. During the assay period, we performed daily plug checks of the females and bedding between ZT2-3.

Fertility assay

For the fertility assessment, virgin 15-17-week old female *Six3*^{NMS} or *Six6*^{NMS} mice were housed with 10-16-week old male mice. The males were left with the females for 76 days. Mating cages were checked every day to see if a litter was born. Days until first litter, how many litters per 76 days, and how many pups per litter were recorded.

Estrous cyclicity

Estrous cyclicity was monitored by vaginal lavage with 20 μl H_2O or saline daily between Zeitgeber time (ZT) 3 and ZT5 for 15-18 days (ZT0 = lights on). The lavage solution was dried on a glass slide and

stained with 0.1-0.5% methylene blue. Cytology was visually examined and scored by two independent observers.

Induced LH surge

Female mice underwent bilateral ovariectomy. Per Bosch et al. [150], four days later, mice received a subcutaneous dose of 0.25 µg estradiol (E2; Cat No. E8875, Sigma Aldrich) in sesame oil (Cat. No. S3547, Sigma Aldrich) between ZT3-4. The following day, mice received a subcutaneous dose of 1.5 µg estradiol in sesame oil between ZT3-4. The following evening (~30 hours after injection), mice were sacrificed at lights out at ZT12. Whole blood was collected, allowed to clot for 90 minutes, and spun at 20 minutes at 2,0000 x g to separate serum. Serum was stored at -80 until analysis.

LH assay

LH was measured using the ultra-sensitive LH assay [151]. . Briefly, serum samples were diluted 1:17 in assay buffer (PBS + 0.2% BSA). The sandwich ELISA was performed using LH 518B7 (UC Davis, Cat. no. 518B7, lot 13; RRID:AB_2665514) as a capture antibody at 1.0 µg/mL and biotinylated 5303 SPRN-5 (Medix Biochemica RRID:AB_2784503) as a detection antibody at 4.379 µg/mL. A standard curve from 1 to 0.0019531 ng/mL was generated using Golden West Biosolutions, catalog No. TLIA1053.03. The plate was analyzed using a Tecan infinite 200 Pro plate reader at 490 nm minus the 650 nm background. A four-parameter logistic curve was generated using MyAssays.com website (http://www.myassays.com/). The intra-assay CV was 7.22%, and the inter-assay CV was 6.21%.

Corticosterone sampling

Pair-housed were allowed to acclimate in chambers on a 12:12 light:dark cycle for two weeks. Tail bleeds were collected every 4 hours for 24 hours into capillary tubes, and serum was collected and stored at -20 C until assay. Approximately 10 µL of tail blood was collected using a capillary (Drummond Scientific, Broomall, PA; # 1-000-0400) from each mouse at each timepoint. To minimize the number of times that the tail was cut for repeated blood collection, cuts from previous timepoints were re-opened with cotton swab soaked in saline. After the 24-hour collection in LD, mice were transitioned to constant

darkness for four weeks before a DD collection. Because the activity rhythms of the mice were not monitored, tail bleeds in DD were collected based on clock time rather than circadian time.

Corticosterone ELISA

Corticosterone was measured from serum using the DetectX Corticosterone ELISA kit (Arbor Assays, #K014-H5) according to manufacturer's instructions. For all samples, 4 µL of serum was diluted 1:400 in dissociation reagent prior to loading on the assay. The plates were visualized using an iMark microplate reader (Bio-Rad) and final concentrations were quantified using a four-parameter logistic curve on MyAssays.com.

Wheel-running behavior

Female and male mice aged 8-12 weeks at the start of the experiment were single housed in cages containing metal running wheels and wheel revolutions were monitored using magnetic sensors as previously described [143]. All cages were contained in a light-tight cabinet with programmable lighting conditions and rooms were monitored for temperature and humidity. Food and water were available *ad libitum* during the entire experiment. After 1-week acclimation to the polypropylene cages (17.8 × 25.4 × 15.2 cm or 33.2 × 15 × 13.2 cm) containing a metal running wheel (11.4 cm diameter or 11 cm diameter, respectively), locomotor activity rhythms were monitored with a VitalView data collection system (Version 4.2, Minimitter, Bend OR) that integrated in 6 minute bins the number of magnetic switch closures triggered by half wheel rotations or full wheel rotations, respectively. Running wheel activity was initially monitored for 2 weeks in a standard 12 h light/12 h dark cycle (LD), whereafter the mice were monitored for 4 weeks in constant darkness (DD), with wheel running data analyzed from weeks 2-4 (14 days) in DD. In some cases, mice were exposed to a two week "skeleton" light paradigm (1h light: 11h dark: 1h light: 11h dark) in between LD and DD; we found no effect of skeleton photoperiod on tau or amplitude, so we excluded it from future experiments. Cage changes were scheduled at 2-4-week intervals. Light intensity varied between 268-369 lux inside the mouse cage with wheel. Wheel running activity was analyzed using ClockLab Analysis (ActiMetrics) by an experimenter blind to experimental group. Circadian period was

analyzed by constructing a least-squares regression line through a minimum of 13 daily activity onsets. No differences were found between male and female mice, therefore they were grouped together for analysis.

Ex vivo tissue recordings of PER2::LUC expression

For circadian rhythm organotypic explant studies, tissues from mice expressing the PER2::LUC circadian reporter were collected and analyzed as previously described [152]. Proestrus PER2::LUC females were placed on LD and euthanized at ZT3-4. The brain, pituitary, ovaries, and uterus were removed immediately and placed in an ice-cold, CO_2 saturated Hank's Balanced Salt Solution (HBSS) for approximately 1 hour. Using a Vibratome (Leica), tissue sections of 300 μm were collected and the indicated brain region was dissected from the slices in ~2x2 mm squares and placed on a 30 mm Millicell membrane (Millipore-Sigma) in a 35 mm cell culture plate containing 1 mL Neurobasal-A Medium (Gibco) with 1% Glutamax (Gibco), B27 supplement (2%; 12349-015, Gibco), and 1 mM luciferin (BD Biosciences). The lid was sealed to the plate using vacuum grease to ensure an air-tight seal. Plated tissues were loaded into a LumiCycle luminometer (Actimetrics) inside a 35˚C non-humidified incubator at ZT6-6.5, and recordings were started. The bioluminescence was counted for 70 seconds every 10 minutes for 6 days (day 1 – day 7 of recording time). PER2::LUC rhythm data were analyzed using LumiCycle Analysis software (Actimetrics) by an experimenter blind to experimental group. Data were detrended by subtraction of the 24 h running average, smoothed with a 2 h running average, and fitted to a damped sine wave (LM Fit, damped). Period was defined as the time in hours between the peaks of the fitted curve.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 10, using Student's t-test, Mann-Whitney U test, one-way ANOVA or two-way ANOVA, followed by post hoc analysis by Tukey or Bonferroni. All data were analyzed as independent measures except for wheel-running activity, which was analyzed via a repeated measures Student's t-test. *p<0.05, **p<0.005, and ***p<0.0005.

Results

Six3, *Six6* and *Nms* expression after SCN neurogenesis

SIX3 and SIX6 share a high degree of homology and are expressed in most NMS neurons within the SCN. Most NMS neurons express both *Six3* and *Six6*, but some NMS cell populations do express one or the other (Fig. 2.1A), indicating the potential differing roles they have in adulthood. To examine the role of *Six3* and *Six6* in the post-developmental regulation of the SCN, we employed an NMS-Cre model. Utilizing tdTomato^{NMS} embryonic tissue, we determined that NMS-Cre has very limited expression within the SCN (Fig. 2.1B-C) in either male or females by E16.5 (N = 3, two females one male). As expression of NMS in the SCN occurs subsequent to the development of the SCN (which occurs from E11-E15), this allowed us to isolate only those effects which occur after SCN neuron cell proliferation is complete and which are unlikely to influence the development of the SCN itself.

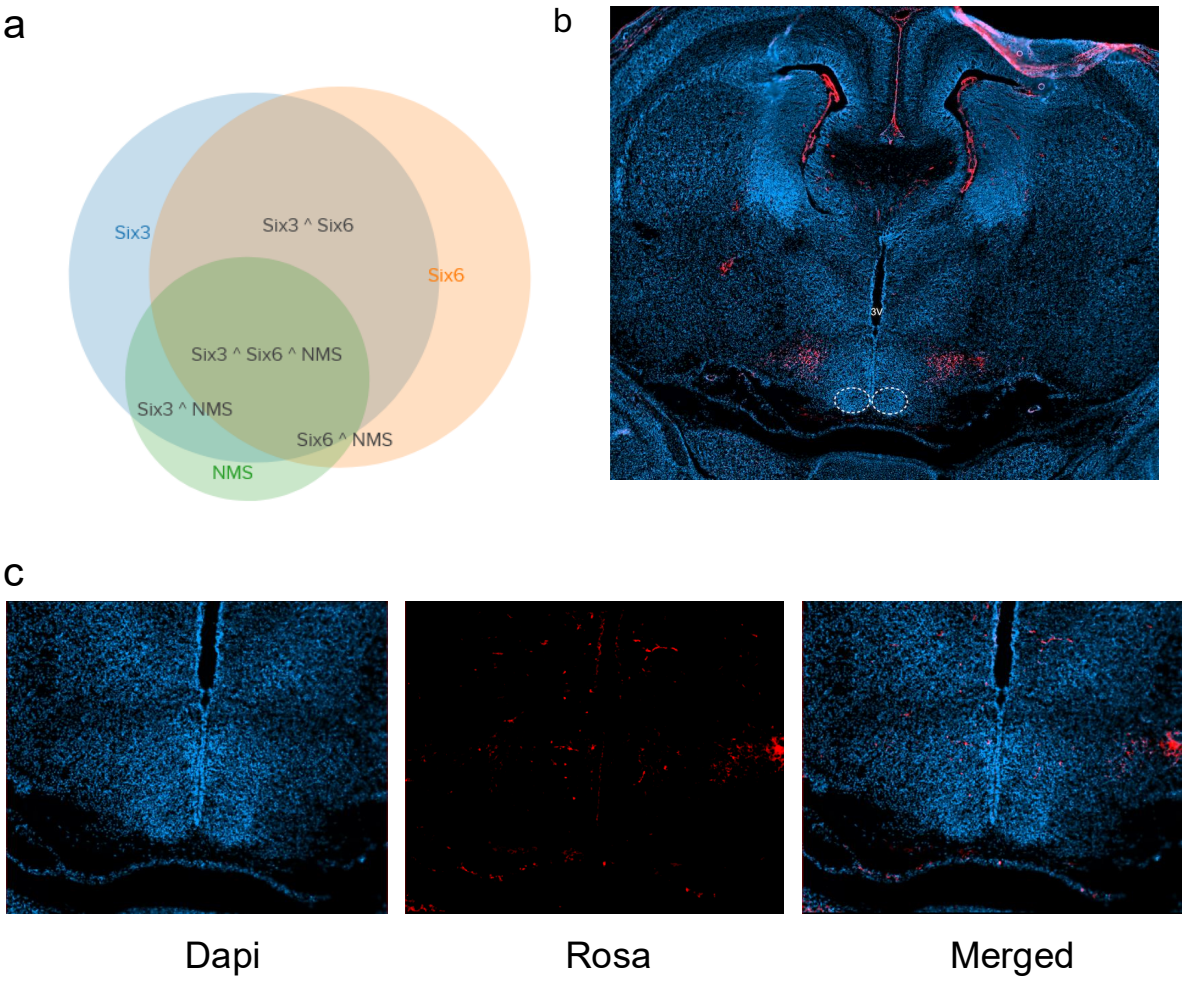

Figure 2.1. *Nms*, *Six3*, and *Six6* expression in the SCN. a) Representation of overlap between *Six3*, *Six6*, and *Nms* in the SCN from published RNA-Seq data [67]. b) Expression of NMS-Cre (as indicated by a tdTomato reporter) at E16.5. Dotted lines outline the SCN. NMS (red) is seen highly expressed far medial to the 3rd ventricle (3V), beyond the SCN. c) Representative closer magnification of the SCN. Images are representative of N = 3.

SIX6 regulates clock genes Per2 and Bmal1.

The molecular clock is a transcription-translation feedback loop known to drive circadian rhythms on a cellular level. To determine if SIX6 regulates genes within the molecular clock and the SCN, we overexpressed SIX6 in the presence of a luciferase reporter for the molecular clock transcripts *Bmal1*, and *Per2*, and peptide transcripts *Avp*, and *Vip* in NIH3T3 cells. In response to SIX6, *Bmal1* luciferase was significantly upregulated (Mann-Whitney Test, $p = 0.0022$) (Fig. 2.2A), whereas *Per2* luciferase was repressed (Mann-Whitney Test, $p = 0.0047$) (Fig. 2.2B), demonstrating that *Six6* can directly regulate the molecular clock. We also found that SIX6 upregulated both *Vip* luciferase (Mann-Whitney Test, $p = 0.0379$) (Fig. 2.2C) and *Avp* luciferase (Mann-Whitney Test, $p = 0.0830$) (Fig. 2.2D) promoter activity. Together, these data suggest that SIX6 plays a regulatory role within the molecular clock by modulating the expression levels of core clock genes and peptides necessary for proper SCN function.

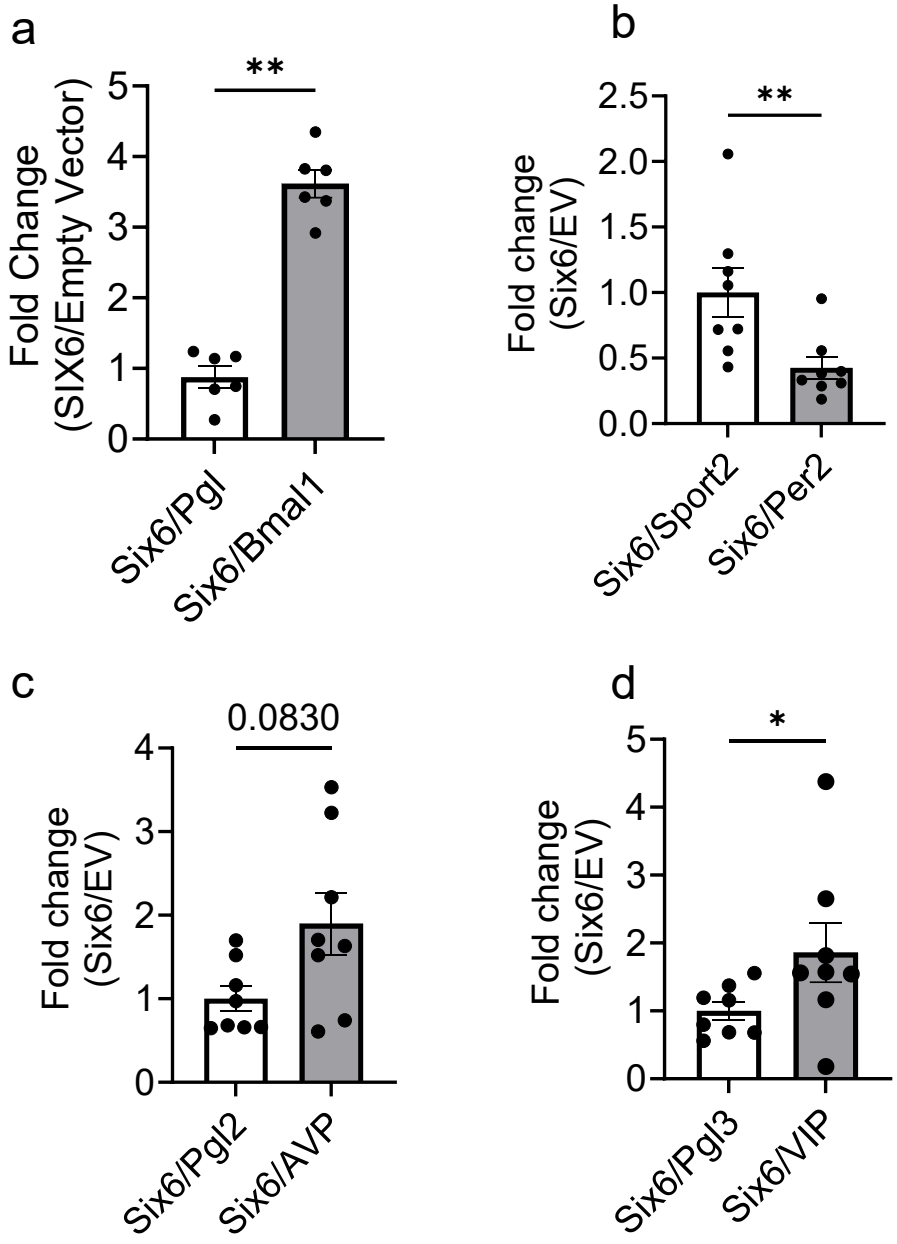

Figure 2.2. SIX6 differentially modulates SCN transcripts *in vitro*. Transient transfections of NIH3T3 cells with a) *Bmal1*-luciferase, b) *Per2*-luciferase, c) *Avp*-luciferase and d) *Vip*-luciferase with *Six6* overexpression vector or empty vector. Data were analyzed using Mann-Whitney U Test. Values were normalized relative to EV. N = 6-8. Data represent mean ± SEM. Significance indicated by *P < 0.05, **P < 0.01.

Loss of *Six3* or *Six6* from NMS-expressing neurons does not impair fertility in conditional knockout females.

LH is vital for proper pubertal development, maintaining the estrous cycle in females, and thus, fertility. LH is regulated upstream by NMS neurons located in the SCN (Vigo et al. 2007; Mori, Miyazato, and Kangawa 2008). To determine how deletion of *Six3* and *Six6* in NMS neurons affects fertility, we assessed female age at vaginal opening (Table 2.1), estrous cycle length (Fig. 2.3A,E), time to first litter (Fig. 2.3B,F), number of litters in 76 days (Fig. 2.3C,G), and pups per litter (Fig. 2.3D,H). We found no differences between either $Six3^{NMS}$ females and controls or $Six6^{NMS}$ females and controls in any of reproductive or fertility markers assayed. There was no difference in time spent in each stage of the estrous cycle (one-way ANOVA, $p > 0.05$).

We then explored whether the timing of the LH surge was disrupted. We have previously found that the loss of *Six3* in Synapsin-containing neurons attenuates the LH surge at lights off [121]. Using an induced surge model in OVX mice, we sacrificed during the AM (ZT2) or at lights off (ZT12, expected time of LH surge). The surge threshold was set using the average of the AM/ZT2 control LH values plus three standard deviations. In this experiment, control mice were a mix of $Six3^{flox/flox}$ and $Six6^{flox/flox}$. We found that 6 of 7 control mice exhibited a PM surge, along with all of the $Six6^{NMS}$ (4/4) and $Six3^{NMS}$ (5/5) mice. The amplitude of the surge was not different among the groups. Therefore, the ability to mount a normally timed LH surge is unaffected in these mice in LD conditions.

Table 2.1. *Six3* and *Six6* in NMS neurons have no effect on puberty onset. Mean ± SEM.

	Control	$Six6^{NMS}$	N, P	Control	$Six3^{NMS}$	N, P
Male Age of preputial Separation (days)	28.50 ± 0.34	28.38 ± 0.50	N = 10, 13 P = 0.86	27.33 ± 1.03	26.50 ± 0.85	N = 6,14 P = 0.0762
Male Weight at preputial separation (g)	13.80 ± 0.74	13.11 ± 0.34	N = 10, 13 P = 0.36	15.72 ± 1.88	14.92 ± 1.78	N = 6, 14 P = 0.3789
Female Age of vaginal opening (days)	29.27 ± 1.04	29.43 ± 1.19	N = 11, 7 P = 0.92	30.93 ± 3.15	30.64 ± 2.54	N = 14,11 P = 0.8048
Female Weight at vaginal opening (g)	12.52 ± 0.45	13.11 ± 0.37	N = 11, 7 P = 0.36	14.51 ± 1.70	14.87 ± 1.23	N = 14,11 P = 0.5543

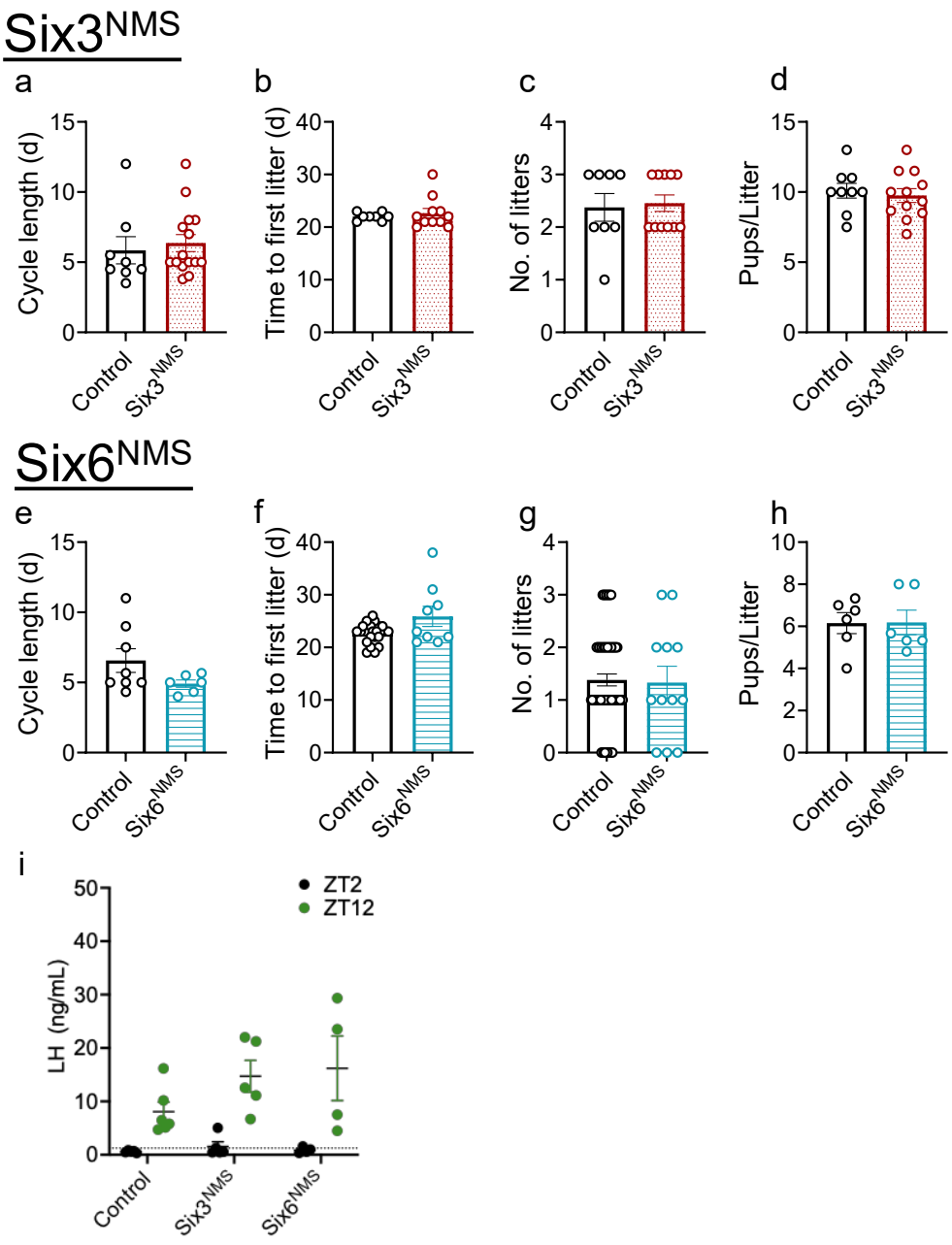

Figure 2.3. Neither Six3^{NMS} nor Six6^{NMS} female mice show changes in fertility measures. When comparing control female mice to Six3^{NMS} mice, there was no significant difference in a) estrous cycle length b) time to first litter c) number of litters in 76 days or d) pups per litter ($p > 0.05$). Similarly, Six6^{NMS} mice showed no difference from controls in e) estrous cycle length f) Time for first litter g) number of litters in 76 days or h) pups per litter ($p > 0.05$). i) no significant differences were found when examining the levels of LH during the pre-ovulatory surge. Two-way ANOVA, $p > 0.05$.

Loss of *Six3* from NMS neurons results in reduced fertility in conditional knock-out males.

NMS neurons within the SCN modulate LH levels in males [153] indicating a role for NMS in male reproduction. To determine whether differences in LH affect postnatal development, we observed pubertal onset in both $Six3^{NMS}$ and $Six6^{NMS}$ male mice. Age and body weight at the time of preputial separation were recorded. No differences were found between either $Six3^{NMS}$ or $Six6^{NMS}$ mice and controls (Table 2.1). After sexual maturity, we examined the number of pups per litter (Fig. 2.4A,D), total sperm (Fig. 2.4B,E), and the percent motile sperm (Fig. 2.4C,F) in both $Six3^{NMS}$ and $Six6^{NMS}$ male mice. $Six3^{NMS}$ males showed a reduction in the percent of motile sperm ($t[10] = 2.632$, $p = 0.0251$), which did not result in a difference in plugging efficiency (time to first plug comparable with controls, t-test, $p > 0.05$). $Six6^{NMS}$ males had no differences in fertility measures. Due the reduced sperm quality of $Six3^{NMS}$ males we examined whether NMS-Cre was expressed in the testis using a $tdTomato^{NMS}$ reporter mouse. We found evidence of sporadic Cre expression in the interstitial cells and spermatids, suggesting that testis-specific knockdown of *Six3* may be occurring in the $Six3^{NMS}$ mice and driving the phenotype (Fig 2.4G).

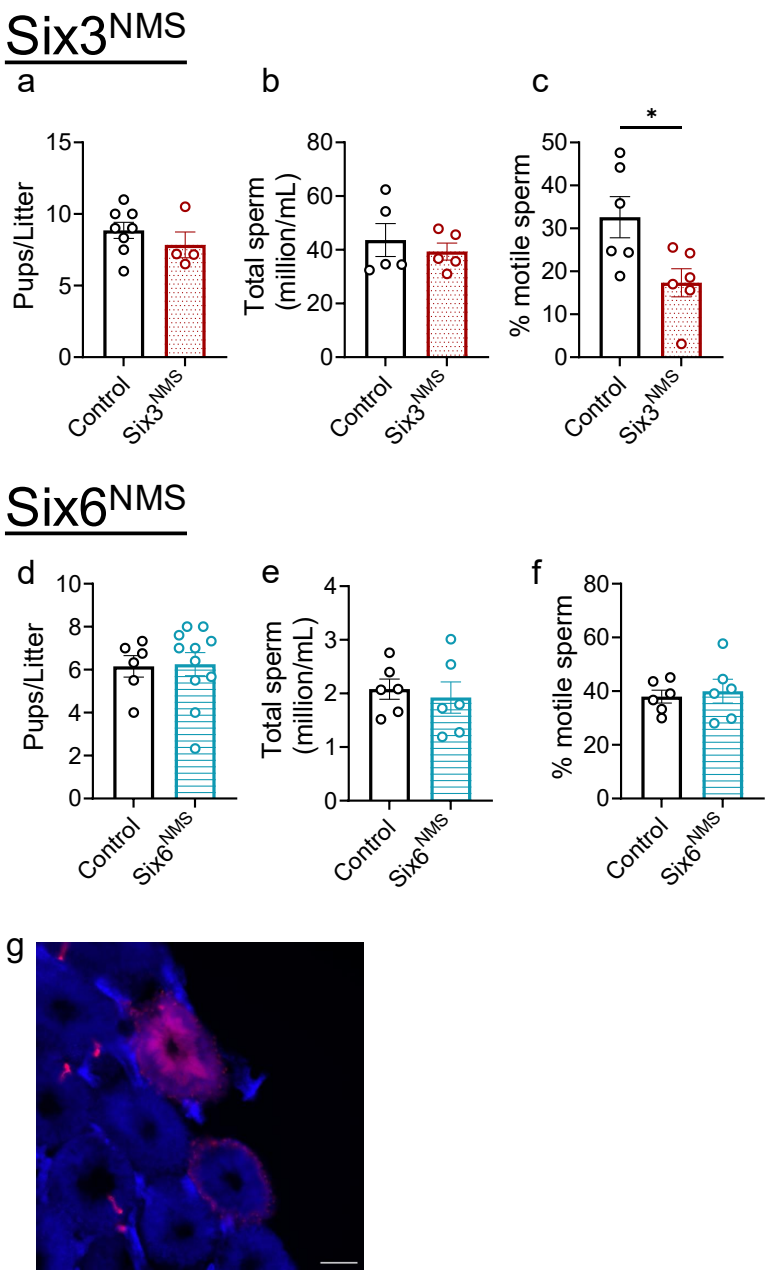

Figure 2.4. *Six3*, but not *Six6*, in NMS cells affects sperm through an unknown mechanism. When comparing control males to Six3^{NMS} mice, there was no significant difference found in a) pups per litter or b) total sperm, however, c) Six3^{NMS} mice had a significant reduction in the percent of motile sperm. When comparing Six6^{NMS} mice to controls, there was no significant difference in d) pups per litter, e) total sperm counts, or f) percent sperm motility *, $p < 0.05$. g) Section of a 26-week-old mouse testis with NMS-Cre reporter expression (tdTomato) counterstained with DAPI (blue). Scale bar = 100 µm.

Loss of *Six3* or *Six6* in NMS neurons does not alter time-dependent hormonal processes.

To determine if other temporally released hormones might be affected, we next monitored cortisol rhythms in $Six3^{NMS}$ mice. After habituation, mice in LD conditions were tail bled every four hours for 24 hours. We found no difference in the amplitude or timing of the cortisol peak in LD conditions (Fig 2.5A). Mice were then allowed to free-run for two weeks in DD conditions before undergoing tail bleeds every 4 hours for 24 hours. Sampling times were not aligned to circadian activity, and due to drifts in the cortisol peak due to differences in free running rhythms, the data are shown as aligned to the peak time (Fig 2.5B). Similar to the LD conditions, within sex, we found no differences in the peak cortisol level or area under the curve. When we examined when the peak cortisol peak occurred in DD, we found it was significantly earlier in $Six3^{NMS}$ mice (Student's t-test, $p = 0.0165$).

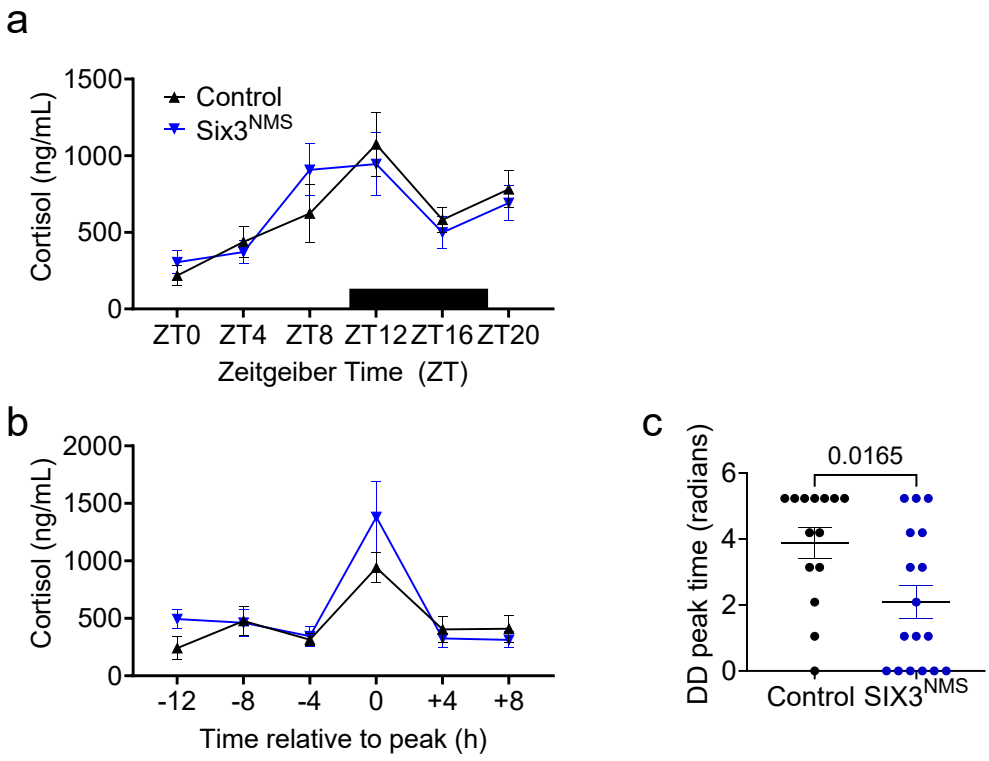

Figure 2.5. Loss of *Six3* in NMS neurons does not affect cortisol amplitude or timing in LD conditions. a) Using serial tail bleeds, there was no significant difference in the cortisol amplitude or peak timing in $Six3^{NMS}$ compared to $Six3^{flox/flox}$ mice in 12:12 light:dark conditions. In constant darkness, b) there was no difference in cortisol peak when aligned by peak time, but the time of peak cortisol c) was significantly different in $Six3^{NMS}$ mice compared to control. $P < 0.05$.

Loss of *Six3*, but not *Six6*, from NMS and VIP neurons shortens free-running period.

We next examined the role of *Six3* and *Six6* in circadian time keeping by measuring locomotor activity. Mice were placed on running wheels in 12h light:12h dark (LD) conditions to establish baseline activity and normal entrainment to light. The mice were then exposed to a constant darkness (DD) paradigm for four weeks to assess changes in endogenous SCN free-running period (Tau). First, we confirmed that the NMS^{cre} mice had no alterations in Tau compared to control littermates (Student's t-test, $p > 0.05$) (Fig. 2.6A). We found that loss of *Six6* from NMS neurons results in comparable period to controls in constant darkness (Student's t-test, $p > 0.05$) (Fig. 2.6B). We confirmed this lack of circadian change with *ex vivo* explants from the SCN, arcuate nucleus, pituitary gland, ovaries, and uterus, which showed no significant differences in PER2::LUC rhythms between PER2::LUC-$Six6^{NMS}$ mice and controls (Table 2.2). Loss of *Six3* from NMS neurons resulted in a shortened Tau compared to controls ($t(31) = 4.096$, $p = 0.0003$) (Uncorrected Fisher's LSD) (Fig. 2.6C).

As NMS neurons overlap extensively with AVP and VIP neurons, we wanted to determine if loss of *Six3* from one of these populations would further parse where *Six3* is acting to affect locomotor rhythms. Upon loss of *Six3* from VIP neurons, we found shortened Tau in the conditional knock out ($t(30) = 3.443$, $p = 0.0017$), whereas loss of *Six3* from AVP neurons resulted in a trend toward shortened Tau in the conditional knock outs ($t(8) = 2.215$, $p = 0.0567$) (Fig. 2.7).

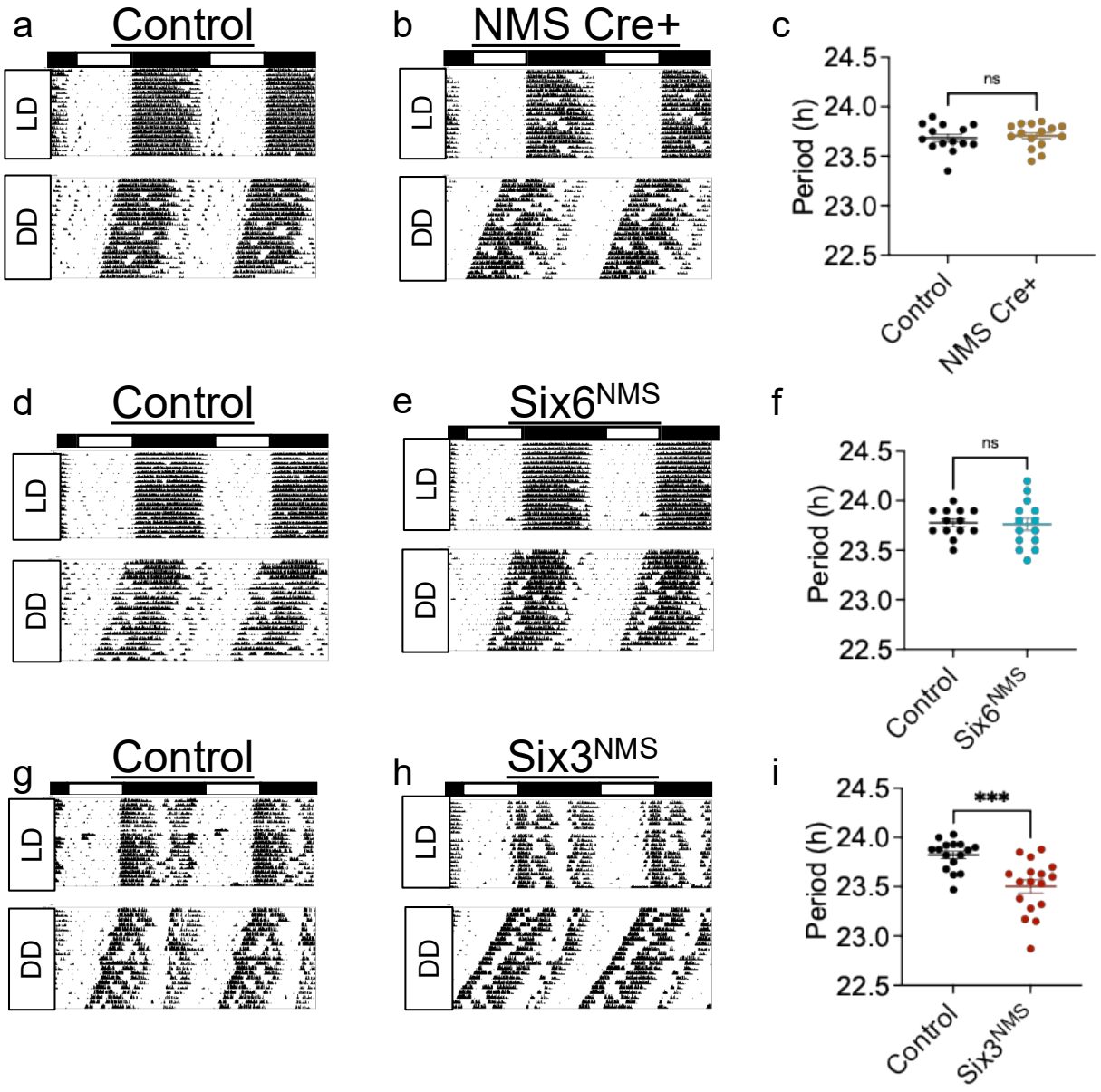

Figure 2.6. *Six3*, but not *Six6* in NMS neurons, regulates locomotor rhythms in constant darkness. Representative actograms from a) control mice compared to b) mice expressing cre-recombinase, c) no significant differences were found in the free-running periods. When comparing the d) control mice to e) $Six6^{NMS}$ mice, f) no significant differences were found in the free-running period. When comparing g) control mice to h) $Six3^{NMS}$ mice, i) $Six3^{NMS}$ mice have a significantly shortened free running period *, $p < 0.05$; **, $p < 0.01$.

Table 2.2. Proestrus female ex vivo explants period as recorded via lumicycle. ZT3-4. Mean ± SEM.

	Control	**Six6^{NMS}**	**P**	**n**
Female SCN	26.61 ± 0.8741	25.47 ± 0.28	0.2471	5,5
Female Arcuate	25.10± 0.6376	24.21 ± 0.45	0.3032	5,6
Female Pituitary	24.90 ± 0.1979	25.50 ± 0.39	0.1830	5,6
Ovary	25.36 ± 0.3071	25.59 ± 0.47	0.6908	6,6
Uterus	25.34 ± 0.3795	26.00 ± 0.45	0.2920	5,5

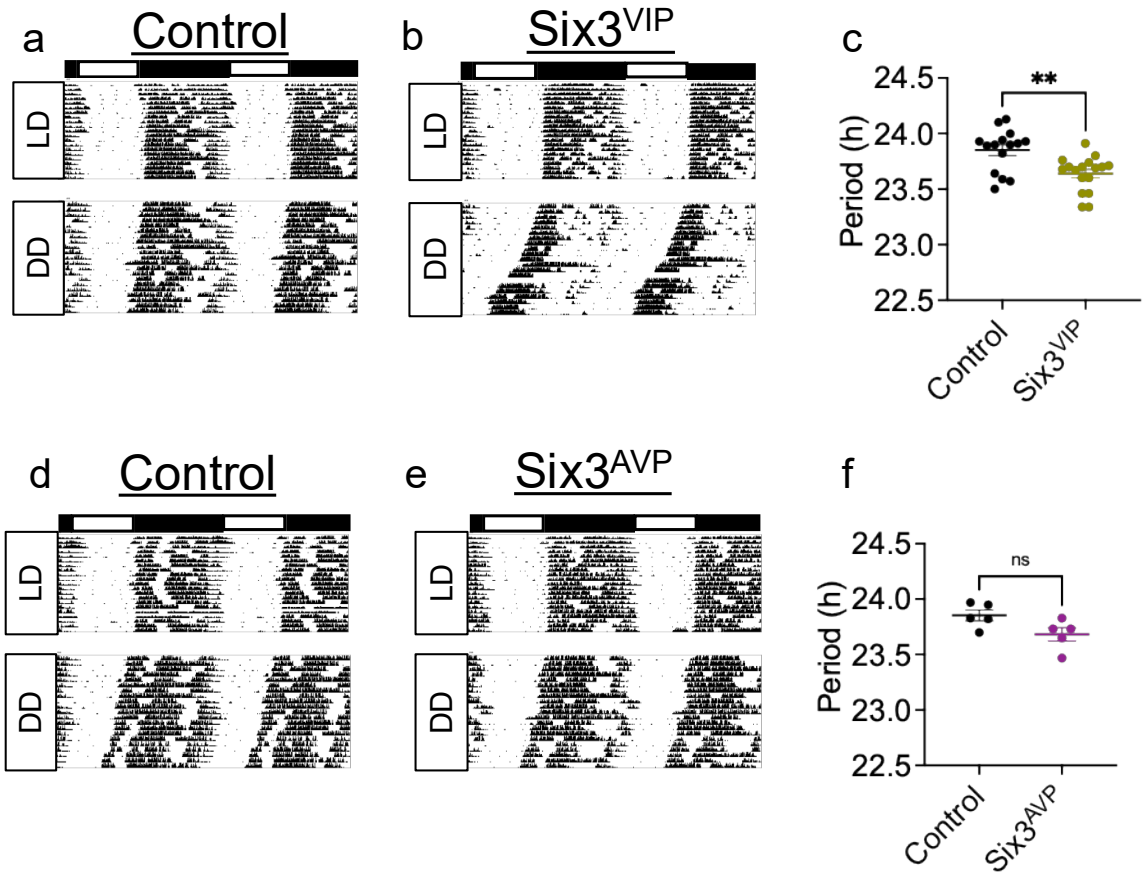

Figure 2.7. *Six3* in VIP neurons regulates locomotor rhythms in constant darkness. Representative actograms from a) a control mouse compared to b) $Six3^{VIP}$ mouse. c) $Six3^{VIP}$ mice have a significantly shortened free running period compared to controls. When comparing d) control mice with e) $Six3^{AVP}$ mice, f) $Six3^{AVP}$ mice trend towards a shortened free-running period. **, $p < 0.01$.

Discussion

Six3 and *Six6* are developmentally essential transcription factors that persist in the adult SCN [122]. The discrete localization of *Six3* specifically to the adult SCN makes the Six3^{cre} mouse an attractive model for targeting SCN neurons. Indeed, using published sc-RNASeq datasets, we found that far more adult SCN neurons express either *Six3* or *Six6* than NMS [67]. However, most *Nms*-containing neurons also express *Six3* and *Six6*, with a minority expressing just *Six3* or *Six6* [67]. Given our findings on the phenotypic differences between the *Six3*^{NMS} and *Six6*^{NMS} animals, this overlap suggests distinct roles of *Six3* and *Six6* within the same population of NMS neurons. Alternatively, *Six3* may be able to compensate for the loss of *Six6*, but not the other way around. Unfortunately, we were unable to validate antibodies to distinguish SIX3 and SIX6 knock-out efficacy, due to the high homology between SIX3 and SIX6. Future experiments using new technology to tease out any compensatory relationship between *Six3* and *Six6* in the discrete neuronal populations would be of great interest.

In our model, we found very low NMS^{cre} expression at E16.5 in the SCN, after SCN neurogenesis [154]. Using NMS^{cre} to knockdown *Six3* and *Six6* later in development resulted in distinct phenotypes from full-body deletion of either *Six3*$^{+/-}$ or *Six6*$^{-/-}$. Previous work has found that deletion of *Six3* in post-proliferative neurons (driven by synapsin-cre) results in disorganized and weaker locomotor rhythms in males and females as measured by χ^2 amplitude [121], [131]. However, we did not find that loss of *Six3* in NMS neurons caused a similar disorganization. In contrast, *Six3*^{SYN} mice had no changes to tau, whereas the *Six3*^{NMS} mice had faster free running periods. These differences may reflect differences in the timing of *Syn*^{cre} and *Six3* recombination between the two populations, or differences in the number or population of SCN neurons targeted by the respective cres.

Full body *Six6*$^{-/-}$ mice display highly variable optic nerve formation and no canonically identifiable SCN. However, *Six6*$^{-/-}$ mice with optic nerves have relatively normal rhythmicity in LD, despite the absence of detectable SCN markers AVP, VIP, or TH [129]. In light of our findings, *Six6* appears more critical prior to or during SCN neurogenesis, and not for circadian rhythm regulation after SCN formation.

Six3 and Six6 in reproduction

In both humans and mice, female reproduction is more sensitive to changes in the circadian system than males, due in part to the precise timing of hormone release required for ovulation [155]. Changes to the circadian system can also impact male reproduction, as seen in altered feeding paradigms and shift work [61], [156]. Our work demonstrates that male reproduction can be mildly impacted by the loss of *Six3*, but not *Six6* from NMS cells. Previous work has shown that *Six3* is required to properly develop the main olfactory epithelium that drives males to smell estrous females [139], and that conditional knockout of *Six3* from kisspeptin neurons within the anteroventral periventricular nucleus (AVPV) results in reduced sperm motility [157]. Recent work has found that NMS neurons from the SCN project directly to kisspeptin neurons in the AVPV [158], providing a potential neurological pathway through which loss of *Six3* from NMS neurons may impact sperm motility. Alternatively, we have also detected NMS^{cre}-mediated recombination in the testis, so a local effect of *Six3* cannot be ruled out. Further work will be needed to determine the exact mechanism through which sperm motility is decreased.

Female fertility was unaffected in $Six3^{NMS}$ and $Six6^{NMS}$ mice. Full body $Six3^{+/-}$ females are subfertile, likely due to abnormal migration of GnRH neurons [142]. Loss of *Six3* in post-proliferative neurons ($Six3^{SYN}$) leads to a disrupted preovulatory LH surge and subfertility in females. Due to the widespread expression of *Syn*, this effect cannot be specifically localized to the SCN [121]. Further, homozygous loss of *Six6* results in subfertility in both sexes [132]. Both $Six3^{NMS}$ and $Six6^{NMS}$ failed to recapitulate the sub- or infertile phenotypes of other *Six3* or *Six6* knockouts or knockdowns, indicating that these genes are not regulating reproduction at the level of the SCN after SCN neurogenesis.

NMS, Six3, and Six6 in Circadian Regulation

The circadian locomotor period was shortened in $Six3^{NMS}$ mice, with a similar shortening in $Six3^{VIP}$ mice, suggesting that the effects seen in Six3NMS mice may be due to the role of Six3 in VIP neurons. NMS neurons often co-express AVP or VIP [67] and make up around 40% of the SCN [99]. It is known that NMS neurons are involved in circadian regulation, though likely not NMS peptide itself. Mice that have NMS neurons selectively silenced in the SCN using tetanus light chain toxin lose rhythmic activity

[100], though full-body NMS knockout mice show no differences in rhythmic activity [159]. Mice in which clock genes are conditionally altered in NMS neurons either through a deletion of *Bmal1* or through insertion of a dominant negative form of *Clock* (clock-Δ19) [98], [100], have disrupted rhythms, as shown by irregular activity in constant darkness. It may be then that the alterations of *Six3* in NMS neurons impacts circadian rhythms indirectly through impacts on the clock genes required for the function of the molecular clock instead of altering NMS peptide indicating that while NMS peptide itself may not be involved in the regulation of circadian rhythms, the activity of neurons which express NMS is critical for circadian rhythm regulation.

Conclusion

Six3 and *Six6* have differing roles in NMS neurons in the regulation of circadian rhythms. Loss of *Six3* from NMS neurons, which occurs after SCN development, results in a mild reproductive phenotype in males and a shortened free-running period in both males and females. However, loss of *Six6* from NMS neurons results in no reproductive or circadian phenotypes. Together, these data show that *Six3* and *Six6*, though related and often compensatory genes, play different functions in the male and female SCN after its development.

Acknowledgements

The authors thank members of the Mellon and Hoffmann laboratories for providing critical review of this manuscript and valuable discussion. We would also like to thank Ichiko Saotome, Chengxian Shi, and Dominique L. M. Gillette for technical assistance

CHAPTER 3: THE TRANSCRIPTION FACTOR VAX1 IN VIP NEURONS OF THE SUPRACHIASMATIC NUCLEUS IMPACTS CIRCADIAN RHYTHM GENERATION, DEPRESSIVE-LIKE BEHAVIOR, AND THE REPRODUCTIVE AXIS IN A SEX SPECIFIC MANNER IN MICE

The following chapter was previously published in Frontiers of Endocrinology [143].

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Author Contributions: All authors provided contributions to study conception and design, acquisition of data or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published. Here are the most important contributions of each author: H.M.H., A.M.Y., B.M.V.L., K.J.T., P.L.M., and M.R.G. designed the study. Data were collected by B.M.V.L., A.M.Y., D.N., K.J.T., H.M.H., J.A.B., B.J., K.J., F.R., D.L.M.G., E.V.H., L.J.C., and L.S. Analysis was carried out by A.M.Y., K.J., F.R., B.M.V.L., K.J.T., L.S., and H.M.H. The manuscript was written by B.M.V.L., A.M.Y., and H.M.H. All authors approve and take final responsibility for this article.

Abstract

The suprachiasmatic nucleus (SCN) within the hypothalamus is a key brain structure required to relay light information to the body and synchronize cell and tissue level rhythms and hormone release. Specific subpopulations of SCN neurons, defined by their peptide expression, regulate defined SCN output. Here we focus on the vasoactive intestinal peptide (VIP) expressing neurons of the SCN. SCN VIP neurons are known to regulate circadian rhythms and reproductive function. To specifically study SCN VIP neurons and to address our hypothesis that abnormal SCN VIP neuron function causes disruption in circadian timekeeping, fertility, and depressive-like behavior, we generated a novel knock out mouse line by conditionally deleting the SCN enriched transcription factor, Ventral Anterior Homeobox 1 (Vax1) in VIP neurons (Vax1flox/flox:VipCre mice; $Vax1^{Vip}$). In accordance with our expected results, we found that $Vax1^{Vip}$ females presented with lengthened estrous cycles, reduced circulating estrogen, and increased depressive-like behavior. Further, $Vax1^{Vip}$ males and females presented with shortened circadian period in locomotor activity and ex vivo SCN circadian period. On a molecular level, the shortening in SCN period was driven, at least partially, by a direct regulatory role of VAX1 on the circadian clock genes Bmal1 and Per2. Interestingly, $Vax1^{Vip}$ females presented with increased expression of arginine vasopressin (Avp) in the paraventricular nucleus, which resulted in increased circulating corticosterone. SCN VIP and AVP neurons regulate the reproductive gonadotropin-releasing hormone (GnRH) and kisspeptin neurons. To determine how the reproductive neuroendocrine network was impacted in $Vax1^{Vip}$ mice, we assessed GnRH sensitivity to a kisspeptin challenge in vivo. We found that GnRH neurons in $Vax1^{Vip}$ females, but not males, had an increased sensitivity to kisspeptin, leading to increased luteinizing hormone release. Interestingly, $Vax1^{Vip}$ males showed a small, but significant increase in total sperm and a modest delay in pubertal onset. Both male and female $Vax1^{Vip}$ mice were fertile and generated litters comparable in size and frequency to controls. Together, these data identify VAX1 in SCN VIP neurons as a neurological overlap between circadian timekeeping, female reproduction, and depressive-like symptoms in mice, and provide a novel insight into the role of SCN VIP neurons.

Introduction

The circadian system is responsible for coordinating circadian and time-of-day signals throughout the body. On a cellular level, circadian rhythms are produced by the molecular clock, a transcription/translation feedback loop that generates 24-hour cell autonomous rhythms [160]–[162]. Alterations in molecular clock function can lead to changes in circadian behaviors and fertility, as shown in transgenic mouse models with a loss of *Bmal1*, a gene required for molecular circadian rhythm generation [44], [163]–[166]. Along with proper molecular clock function, the suprachiasmatic nucleus (SCN), located in the ventral hypothalamus, is the central pacemaker of the brain and serves to coordinate external timing signals and physiological processes throughout the body. Importantly, the SCN requires a finely balanced neuropeptide expression to maintain circadian rhythms [81]. Correct levels of vasoactive intestinal peptide (VIP), along with other neuropeptides such as arginine vasopressin (AVP), somatostatin, and gastric releasing peptide, are required for SCN output controlling synchrony both within and downstream of the SCN, including behavioral and tissue level circadian rhythms. Both the molecular clock and neuropeptide expression must function correctly to maintain strong and synchronized circadian rhythms [72], [167]–[169]. SCN VIP-expressing neurons play an important role in aligning SCN function to the time of day by relaying photic information from the optic nerve to generate synchrony among SCN neurons and SCN output [170], [171].

One of the well-established downstream effects of SCN VIP neurons is the regulation of neuroendocrine function, including the regulation of gonadal sex steroids needed for female fertility [77], [165], [172]–[174]. In the male, the role of VIP neurons with regards to reproductive function is less clear. It has been suggested that VIP may be involved in testicular health [175], [176], but no reproductive phenotype has been reported in any *Vip* knock-out mouse line to our knowledge. Additional studies have demonstrated that impaired SCN function or disrupted circadian rhythms in the SCN or periphery do not, or only modestly, disrupt male fertility, supporting the idea that male fertility is more robust than female fertility and often resilient to circadian disruption (Dolatshad et al., 2006; Li et al., 2018; Hoffmann, Pandolfi, et al., 2019; Hoffmann et al., 2021; Meadows et al., 2022). In contrast, female fertility relies on

precise synchronization between hormone release and peripheral tissue function [126], [155], [179]. To regulate the reproductive axis, VIP neurons project directly and indirectly through SCN AVP neurons and kisspeptin neurons [180]–[183] to gonadotropin-releasing hormone (GnRH) neurons. GnRH is released through both pulse and surge modes into the median eminence, promoting the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The timing of the LH surge is particularly important in females at both the level of the hypothalamus and the ovary for optimal ovulation [184], [185]. This surge requires coordinated input to GnRH neurons from both the SCN and kisspeptin neurons. In the ovary, the molecular clock is required in ovarian theca cells to increase LH receptor expression around the time of day of the LH surge, facilitating ovulation [58].

Given their importance for both circadian timekeeping and reproductive health, the role of VIP neurons in the SCN is an important area of investigation in the context of female reproduction. Previous work has shown that changes in VIP alter circadian rhythms in mice [48], [76], [186], with full-body VIP knock-out female mice having disruptions in both the circadian and reproductive systems, demonstrating the importance of VIP in both processes [77]. Such changes in VIP can also negatively impact reproductive hormone release through dysregulation of the required neurocircuitry for LH and FSH release [85], [187], [188], which can, in turn, lead to dysregulation of female reproductive sex steroids [189], an imbalance that can have negative effects on both reproductive function and mood [190], [191]. Evidence supports that disrupted circadian rhythms and changes in estrogen and progesterone might be contributing factors to depressive symptoms in humans [188], [192]. Together this evidence indicates a potential shared origin between circadian deregulation, reproductive deficits, and mood changes at the level of VIP neurons.

In this study, we hypothesized that abnormal SCN VIP neuron function causes disruption in circadian timekeeping, fertility, and depressive-like behavior. We deleted the SCN-enriched transcription factor, Ventral anterior homeobox 1 (VAX1) within the VIP neurons of mice. Our previous work has shown that VAX1 is required for SCN development [14], [120] and maintains a function in late development of the SCN and VIP neurons, where it is required for VIP expression, SCN output and female fertility [121]. Due to the specific overlap between VAX1 and VIP in the SCN, shown here, the use of $Vax1^{flox/flox}$:Vip^{Cre}

mice provides a model to specifically investigate how weakened, but not ablated, SCN VIP neuron function regulates reproductive function, SCN circadian output, and mood. Identifying the shared genetic underpinnings of the association of reproductive and mood disorders is a required first step towards future development of efficient strategies to improve hormone related mood disorders. We expect that VAX1 in postnatal VIP neurons is required for VIP neuron function, where loss of VAX1 in VIP neurons causes weakened SCN output, leading to female, but not male, subfertility and increased depressive-like symptoms.

Materials and Methods

Mouse breeding

All animal procedures were performed according to protocols approved by the University of California, San Diego Institutional Animal Care and Use Committee and the Institutional Animal Care and Use Committee of Michigan State University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals [193] (National Research Council, 2011). Mice were maintained on a light/dark cycle of LD [12 h light, 12 h dark, average light intensity ~150-350 lux within the cage]. Based on the newly proposed light reporting method by [194], [195], we determined the relative perception of light by mice using the mouse α-opics equivalent daylight illuminance (EDI). We calculated that the α-opics for our experimental mice on LD to be melanopsin = 43.2 ± 16.9 lux, rod = 51.6 ± 19.8 lux, s-cone = 0.03 ± 0.01 lux, and m-cone = 57.1 ± 21.7 lux. Lights ON = ZT0 (6:00), lights OFF = ZT12 (18:00)], Zeitgeber time (ZT). $Vax1^{flox/flox}$ [$Vax1^{tm1c(KOMP)Mbp}$, MGI: 5796178] [196], were crossed with mice heterozygous for the $Vip^{Cre/wt}$ allele [JAX #010908]. Period2::Luciferase (PER2::LUC) mice were purchased from JAX (strain B6.129S6-Per2tm1Jt/J, JAX #006852) and crossed with the offspring of $Vax1^{flox/flox}$ and $Vip^{Cre/wt}$ mice. We did not detect any significant differences in mice heterozygous for the $Vip^{Cre/wt}$ allele as compared to $Vax1^{flox/flox}$ mice, thus we pooled the $Vax1^{flox/flox}$ and $Vip^{Cre/wt}$ control groups into one control group, referred to as Ctrl. Genotyping primer sequences were as follows: Vax1-wtF: CCAGTAAGAGCCCCTTTGGG, Vax1-floxF: GCCGGAACCGAAGTTCCTA; Vax1-R: CGGATAGACCCCTTGGCATC; CreF: GCATTACCGGTCGTAGCAACGAGTG, CreR: GAACGCTAGAGCCTGTTTTGCACGTTC. Mice

were kept on a C57BL/6J background and were screened for germline recombination. Mice with germline recombination were excluded from the studies. VIP-tdTomato mice were generated by crossing $Vip^{Cre/wt}$ mice with Rosa-tdTomato reporter mice (JAX# 007914). Mice were euthanized by cervical dislocation followed by decapitation, in accordance with AVMA guidelines.

Wheel-running behavior

Female and male mice aged 8-12 weeks at the start of the experiment were single housed in cages containing metal running wheels and wheel revolutions were monitored using magnetic sensors. All cages were contained in a light-tight cabinet with programmable lighting conditions and rooms were monitored for temperature and humidity. Sylvania T8 32-Watt 4100K fluorescent bulbs (F032/841/ECO) were used to provide light to the cabinets. Food and water were available *ad libitum* during the entire experiment. After 1-week acclimation to the polypropylene cages (17.8 × 25.4 × 15.2 cm or 33.2 × 15 × 13.2 cm) containing a metal running wheel (11.4 cm diameter or 11 cm diameter, respectively), locomotor activity rhythms were monitored with a VitalView data collection system (Version 4.2, Minimitter, Bend OR) that integrated in 6 minute bins the number of magnetic switch closures triggered by half wheel rotations or full wheel rotations, respectively. Running wheel activity was initially monitored for 2 weeks in a standard 12 h light/12 h dark cycle (LD). Subsequently, mice were monitored for 4 weeks in constant darkness (DD), with wheel running data analyzed from weeks 2-4 (14 days) in DD. Cage changes were scheduled at 3-week intervals. Light intensity varied between 268-369 lux inside the mouse cage with wheel. Wheel running activity was analyzed using ClockLab Analysis (ActiMetrics) by an experimenter blind to experimental group. Circadian period was analyzed by constructing a least-squares regression line through a minimum of 13 daily activity onsets. Daily onset and offset of activity, defined as a period of 5 h of activity following 5 h of inactivity (onset) or a period of 5 h of inactivity following 5 h of activity (offset), were used to calculate the length of the active phase (alpha). Chi^2 periodograms were generated for periods from 0 to 36 h, with significance set at 0.001. Activity profiles were generated for weeks 2-4 in DD using the estimated chi^2 periodogram tau for the same time period. Total daily counts for mice on wheels with 2 sensors were calculated over 24 h, during both LD and DD.

Porsolt Forced Swim Test

Female mice aged 9-13 weeks were single housed for two weeks on LD, then placed in a transparent 7 by 24 inch cylinder filled 2/3 full with 20-21°C water. Swim tests were completed during the mice active phase and started at ZT13. Mice were recorded in dim red light (5 lux, α-opics: melanopsin = 0.03, rod = 0.20, s-cone = 0, m-cone = 0.54, Supplementary Figure 1C, F) for 6 minutes before they were removed from the water, dried with a cloth, and returned to their cages for one hour before euthanasia and blood collection from the abdominal aorta. Videos were scored by an observer blinded to the experimental group using the sampling method where the mouse is determined to be either floating or swimming every 30 seconds for 5 minutes, with the first minute of each video going unscored and serving as an adjustment period. The percent of intervals where the mouse was observed to be floating is reported [197].

Estrous cyclicity, sperm count, and fertility assessment

For the fertility assessment, virgin 8-12-weeks old male and female Ctrl, and $Vax1^{Vip}$ mice were housed with opposite sex Ctrl mice [198], [199]. The number of litters and the number of pups per litter were recorded over a period of 4 months, as described previously [198]. Estrous cyclicity was monitored by vaginal lavage with 20 µl H_2O daily between ZT3 and ZT5 for 16-18 days. The lavage solution was dried on a slide and stained with 0.5% methylene blue. Cytology was visually examined and scored. Ovary and uterus weights were collected after euthanasia in diestrus. Following euthanasia in males, testes and epididymis were collected and weighed. Sperm was collected from epididymis of male mice in M2 media (Sigma #M7167). Epididymis was cut in half and sperm were expelled by gently pressing down on the epididymis and then left in M2 media at room temperature for 15 min. The numbers of total and motile sperm were counted from a 1:10 dilution of the M2 media containing sperm by using a hemocytometer. The second epididymis was cut into small pieces and left 15 minutes at room temperature in M2 media. The solution was homogenized frequently to help liberate the sperm. The solution was filtered using a cell streamer (70 µm, Falcon #352350) and sperm were diluted 1:10 with MQ before counting total number of sperm heads.

Pubertal onset

Pubertal onset was established by visual inspection of preputial separation (PPS) in males and vaginal opening (VO) in females, as described previously [198]. Body weight was recorded daily until pubertal onset was observed.

Immunohistochemistry staining

Tissues were collected between ZT3 and ZT5 from adult male and proestrus female mice on LD light cycle and fixed overnight at 4˚C in 60% ethanol, 10% formaldehyde, and 10% glacial acetic acid. Tissues were washed in 70% ethanol and embedded in paraffin. Single immunohistochemistry on 10 μm coronal brain sections embedded in paraffin was performed as previously described (Hoffmann, Pandolfi, et al., 2019). The primary antibody was rabbit anti-VIP (Immunostar #20077, 1:1000, RRID:AB_572270). Sections were incubated in 1:300 secondary anti-rabbit IgG (Vector Laboratories, #BA-1000). Secondary antibodies were purchased from Vector labs, and colorimetric VIP (purple staining) and DAB (brown staining) assays (Vector laboratories) revealed the primary antibodies. VIP staining was quantified automatically in six sections evenly distributed between Bregma -0.22 and -0.58 by the Lionheart (BioTek Lionheart FX Automated Microscope) Gen5 software (version 3.10 BioTek) as previously described [121].

$Vip^{Cre/wt}$:Rosa-tdTomato$^{+/-}$ mice (n=4; 2 female and 2 male) were sacrificed between ZT4-6 at 6 weeks of age and brains immersed in 4% PFA overnight at 4°C. Brains were transferred to 30% sucrose until sectioned 40 μm thick with a cryostat. Sections were stored in cyroprotectant at -80°C. Prior to staining, sections were washed overnight in PBS at 4°C. Sections underwent antigen retrieval for 20 minutes in citrate buffer, followed by a wash and blocking for 30 minutes at room temperature using an Avidin/Biotin blocking kit (Vector Labs) with 5% normal goat serum. Sections were briefly washed and then stained following the protocol for the Mouse on Mouse Basic Immunodetection kit (Vector Labs) using a mouse anti-VAX1 (1:100; Origene RRID:AB_2941013). Slices underwent Vectastain ABC kit (Vector Labs) followed by TSA treatment (Akoya Biosciences) for 10 minutes and finally streptavidin-conjugated secondary (1:200) for 30 minutes. Slices were mounted, air-dried, and coverslipped with Prolong Gold with DAPI (ThermoFisher). Slices were imaged on a Nikon Eclipse Ti2-E using a Lumencor SpectraX LED and

acquired using a DS-Qi2 CMOS camera. One SCN section per animal was analyzed using QuPath [200]. All image manipulations were applied homogenously to the entire image.

Multiplex in situ hybridization assay

To examine *Vip*, *Avp*, *Nms*, and *Vax1* mRNA when adult male and female hormones are most comparable, brains were collected at ZT4-8 in young mice and adult males and diestrus females. To examine *Avp*, *Vip*, and *Bmal1* mRNA around the time of the LH surge in Ctrl and $Vax1^{Vip}$ mice, brains were collected at ZT13-16 in proestrus females. Multiplex *in situ* hybridization detection of mouse (*Mus musculus*) mRNAs was performed with RNAscope® LS Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, cat no. 322800) for 3-plex assay in addition to RNAscope® LS 4-Plex Ancillary Kit (Advanced Cell Diagnostics, cat no. 322830) for 4-plex assay following vendor's standard protocol for FFPE tissue sections with minor modifications. RNAscope® assays were performed on a Leica Bond autostainer as described [201] with the following probes: RNAscope® 2.5 LS Probe – Mm-Arntl (also known as Bmal1) [aryl hydrocarbon receptor nuclear translocator-like (Arntl) transcript variant 1 mRNA, cat no. 438748-C1] or RNAscope® 2.5 LS Probe - Mm-Vax1 mRNA – [musculus ventral anterior homeobox 1 (Vax1), cat no. 805108-C1]; RNAscope® 2.5 LS Probe – Mm-Avp-C2 [arginine vasopressin (Avp) mRNA, cat no. 401398-C2]; RNAscope® 2.5 LS Probe – Mm-Vip-C3 [vasoactive intestinal polypeptide (Vip) mRNA, cat no. 415968-C3]; and RNAscope® 2.5 LS Probe – Mm-Nms-C4 [neuromedin S (Nms) transcript variant 1, cat no. 472338-C4]. Stock Mm-Nms-C4 probe was diluted at 1:50 in pre-diluted C1 probe as recommended by the vendor, whereas stock Mm-Avp-C2 and Mm-Vip-C3 were further diluted to 1:100 in appropriate pre-diluted C1 probe due to saturating signal in pilot experiment. Tissue slides were counterstained with DAPI and scanned with Aperio Versa imaging system with 20X objective with customized narrow-width band excitation and emission filter cubes as described [201]. The Aperio Cellular IF Algorithm (Leica Biosystems, No: 23CIFWL) was used for automated cell enumeration and segmentation based on nuclear DAPI staining. Cells were classified based on the expression levels of one or more mRNAs. In images taken at P2, *Vax1* staining was oversaturated, so they were re-imaged for proper

mRNA visualization. Representative images at P2 were displayed with increased contrast, applied to all channels to compare with P10 and P60 in Figure 3.1.

GnRH and Kisspeptin Challenges and Hormonal Assays

Hormonal challenges were done using kisspeptin and GnRH intraperitoneal (i.p.) injections at ZT3-4. Kiss-10 (catalog #42-431, Batch 7A, Fisher Science, 30 nmoles/mouse) was injection to males or diestrus females and blood was collected from the mouse from the tail vein before (time 0) and after i.p. injection at time points 5, 10, 15, 30, and 45 minutes. Tail blood was collected before (time 0) and 10 minutes after i.p. injection of GnRH (Millipore Sigma, catalog #L7134, 1 μg/kg dose). For all other serum hormone analyses, mice were killed by cervical dislocation and blood collected from the abdominal aorta between ZT3 and ZT6. Blood was allowed to clot for 1 hour at room temperature, then centrifuged (room temperature, 15 minutes, 2,600× g).

Serum was collected and stored at -80°C before analysis for estradiol and testosterone at the Center for Research in Reproduction, Ligand Assay, and Analysis Core, University of Virginia (Charlottesville), by Luminex analysis for LH and FSH on MILLIPLEX MAP Mouse Pituitary Magnetic Bead Panel (Millipore Sigma #MPTMAG-49k) or a competitive enzyme-linked immunosorbent assay (ELISA) kit (EIACORT, ThermoFisher) for corticosterone. Coefficients of variance (CVs) were based on the variance of samples in the standard curve run in duplicate. Reportable range: estradiol: 3–300 pg/ml, CV = <20%; T: lower detection limit: 9.6 ng/dl, CV < 10%; LH: lower detection limit: 5.6 pg/ml, CV < 15%; FSH: lower detection limit: 25.3 pg/ml, CV < 15%; corticosterone: lower detection limit: 0.87 μg/dl, CV = <20%. Samples were run in singlets.

Ex vivo tissue recordings of PER2::LUC expression

For circadian rhythm organotypic explant studies, tissues from mice expressing the PER2::LUC circadian reporter were collected and analyzed as previously described [152]. Male and proestrus and diestrus PER2::LUC females were placed on LD and euthanized at ZT3-4 via isoflurane inhalation and cervical dislocation. The brain was removed immediately and placed in an ice-cold, CO_2 saturated Hank's Balanced Salt Solution (HBSS) for approximately 1 hour. Using a Vibratome (Leica), coronal brain sections

of 300 μm were collected and the SCN was dissected from the slices in ~2x2 mm squares and placed on a 30 mm Millicell membrane (Millipore-Sigma) in a 35 mm cell culture plate containing 1 mL Neurobasal-A Medium (Gibco) with 1% Glutamax (Gibco), B27 supplement (2%; 12349-015, Gibco), and 1 mM luciferin (BD Biosciences). The lid was sealed to the plate using vacuum grease to ensure an air-tight seal. Plated tissues were loaded into a LumiCycle luminometer (Actimetrics) inside a 35˚C non-humidified incubator at ZT6-6.5, and recordings were started. The bioluminescence was counted for 70 seconds every 10 minutes for 6 days (day 1 – day 7 of recording time). PER2::LUC rhythm data were analyzed using LumiCycle Analysis software (Actimetrics) by an experimenter blind to experimental group. Data were detrended by subtraction of the 24 h running average, smoothed with a 2 h running average, and fitted to a damped sine wave (LM Fit, damped). Phase was defined as the time of the first peak of the fitted curve. Period was defined as the time in hours between the peaks of the fitted curve. Amplitude is defined as the value of the second peak and phase is defined as the time of first peak. Data from proestrus and diestrus female SCN recordings were pooled as no significant differences in PER2::LUC period or amplitude were found.

Cell culture and transient transfections

NIH3T3 (American Type Culture Collection) and COS-1 (American Type Culture Collection) cells were cultured in DMEM (Mediatech), containing 10% fetal bovine serum (Gemini Bio), and 1x penicillin-streptomycin (Life Technologies/Invitrogen) in a humidified 5% CO_2 incubator at 37˚C. For luciferase assays, NIH3T3 cells were seeded into 24-well plates (Nunc) at 30,000 cells per well. For electrophoretic mobility shift assays (EMSA) COS-1 cells were plated at 1.5 million cells/10 cm dish. Transient transfections for luciferase assays were performed using PolyJet™ (SignaGen Laboratories, Rockville, MD), whereas Fugene was used for plasmid overexpression for EMSA, following manufacturer's recommendations. Transfection of cells was performed 48 h after the cells were plated. COS-1 cells were transfected with Vax1/DKK-Flag or CMV6/DKK-Flag overexpression plasmids (20 ng/well, Origene Technologies, Rockville, MD) and harvested at sub-confluency 48–56 h after transient transfections in 10 cm dishes (Nunc). Transient transfections for luciferase assays were done following manufacturer's

recommendations. NIH3T3 cells for luciferase assays were co-transfected with 150 ng/well of Bmal1-luciferase or Per2-luciferase reporters, 100 ng/well thymidine kinase-β-galactosidase reporter plasmid, which served as an internal control [198], as well as mouse Vax1/pCMV6 overexpression plasmid (20 ng/well, Origene Technologies, Rockville, MD), or its empty vector control (pCMV6). To generate the Bmal1-luciferase plasmid the Bmal1 sequence between -966 bp to +140 bp from the *Bmal1* transcriptional start site was excised from the pABpuro-BluF plasmid (Addgene, Plasmid #46824) with PCR primers (F: gggctacaacagaacaactaac, R: taaacaggcacctccgt). The PCR product was inserted into the pGL3-basic backbone between the Mlu-HF and XhoI sites using the Quick Ligation Kit (New England Biolabs). Site directed mutagenesis of the homeodomain binding sites (ATTA and ATTA-like) in the mouse Bmal1-luciferase plasmid was performed using the NEB Q5 Site-Directed Mutagenesis Protocol (New England Biolabs Inc.), following manufacturer's instructions. Primers for NEB Q5 site-directed mutagenesis were designed using the NEB Base Changer (Table 1). To equalize the amount of DNA transfected into cells, we systematically equalized plasmid concentrations by adding the corresponding inactive plasmid backbone. Cells were harvested 24 h after transfection in lysis buffer [100 mM potassium phosphate (pH 7.8) and 0.2% Triton X-100]. Luciferase values were normalized to β-galactosidase values to control for transfection efficiency. Values were further normalized by expression as fold change compared to pGL3 control plasmid, as indicated in the figure legends. Data represent the mean ± SEM of at least three independent experiments done in duplicate and triplicate.

Cytoplasmic and nuclear extracts and Electrophoretic Mobility Shift Assay (EMSA)

COS-1 cells were scraped in hypotonic buffer (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail; Sigma-Aldrich) and left on ice to swell. Cells were lysed and nuclei were collected by centrifugation (4°C, 1700 *g*, 4 minutes). Nuclear proteins were extracted on ice for 30 minutes in hypertonic buffer [20 mM HEPES, pH 7.9, 20% glycerol, 420 mM KCl, 2 mM $MgCl_2$, 10 mM NaF, 0.1 mM EDTA, 0.1 mM EGTA, 1x protease inhibitor cocktail (Sigma-Aldrich), and 1 mM phenylmethylsulfonylfluoride]. Debris was eliminated by centrifugation (4°C, 20,000 *g*, 10 min), and supernatant was snap-frozen and stored at -80°C.

Oligonucleotide probes are listed in Table 1. All synthetic oligonucleotides were made by IDT (San Diego, CA). Annealed double-stranded oligonucleotides (1 pmol/µl) were end-labeled with T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA) and [γ^{32}P]ATP (7000 Ci/mmol; MP Biomedicals, Solon, OH). Probes were purified using Micro Bio-Spin 6 Chromatography Columns (Bio-Rad). Binding reactions contained 2 µg nuclear protein and 1 fmol of labeled probe in 10 mM HEPES (pH 7.9), 25 mM KCl, 2.5 mM MgCl2, 1% glycerol, 0.1 % Nonidet P-40, 0.25 mM EDTA, 0.25% BSA, 1 mM dithiothreitol, and 350 ng poly(dI-dC). For super-shift experiments, 2 µg mouse anti-DKK (Flag antibody, Origene #TA50011) or 2 µg of normal mouse IgG (Santa Cruz Biotechnology, #sc2025) were added to the reaction. Samples were incubated for 20 minutes at room temperature before loading on a 5% non-denaturing polyacrylamide gel in 0.25x Tris-borate EDTA buffer. Gels were run for 2 h at 200 V, dried under vacuum and exposed to film for 2-5 d at room temperature.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8, using Student's t-test, one-way ANOVA or two-way ANOVA, followed by *post hoc* analysis by Tukey or Bonferroni as indicated in figure legends, with $p < 0.05$ to indicate significance. All data were analyzed as independent measures except for wheel-running activity, which was analyzed via a two-way repeated-measures ANOVA. PER2::LUC timing of first peak phase relationships were analyzed in R via a Circular Analysis of Variance High Concentration F-Test, with a corrected confidence level of $p < 0.01667$ to account for family-wise error.

Results

Characterization of Vax1 expression in Vip, Avp, and Nms neurons in the male and female SCN

Vax1 is highly expressed in the developing mouse SCN and becomes refined to the hypothalamus, primarily in the SCN in the early postnatal period [49], [121]. Although conditional deletion of *Vax1* in late neuronal development using the Synapsin^{Cre} allele reduced VIP expression in the adult SCN [121], it remains unknown if all VIP expressing neurons co-express VAX1 and how postnatal deletion of *Vax1* in VIP neurons impacts VIP expression. Because VAX1 is highly expressed in the developing SCN, we first asked how VAX1 expression changed after birth and into adulthood in males and females. To answer this,

we performed multiplex RNAscope® assay at postnatal day 2 (P2), P10, and P60 (adult) at ZT5 in males and females. We found that all SCN *Vip*-expressing cells at P2 co-express *Vax1* [Figure 3.1A, B, G; male (n = 2) 99.4 ± 0.6%, female (n = 2) 100% ± 0%], a pattern maintained at P10 [Figure 3.1C, D, I; male (n = 4) 99.98 ± 0.021%, female (n = 4) 99.91 ± 0.09%], and P60 [Figure 3.1E, F, K; male (n = 3) 98.80 ± 1.08%, female (n = 4) 100 ± 0%]. In addition to the high co-expression with *Vip*, *Vax1* is highly expressed throughout the SCN at P2, P10, and P60 (Figure 3.1A-F), where both *Avp* and *Nms*-expressing cells also exhibited full overlap with *Vax1* in both sexes (Figure 3.1G, I, K). Interestingly, we found a sex difference in the number of cells expressing *Avp* at P60, where females had fewer *Avp*+ cells compared to males [Figure 3.1L; n = 7, $t(5) = 5.671$, $p = 0.0024$], a difference that was not present prior to puberty (P10). Although we did not see a significant sex difference in the number of *Vip* neurons at any age, the concentration of *Vip*, as evaluated through *Vip* probe signal intensity, was significantly lower in females than males at P10 [$t(3) = 6.01$, $p = 0.037$, not shown] and trended lower at P60 [$t(5) = 2.47$, $p = 0.16$, not shown]. To determine if this modest sex difference in *Vip* concentration translated to a sex difference in peptide levels, we performed IHC in adult male and female brains. Adult female mice had a significant reduction in the intensity of VIP peptide in the SCN [Figure 3.2A, B, $t(11) = 3.874$, $p = 0.0026$] as compared to males.

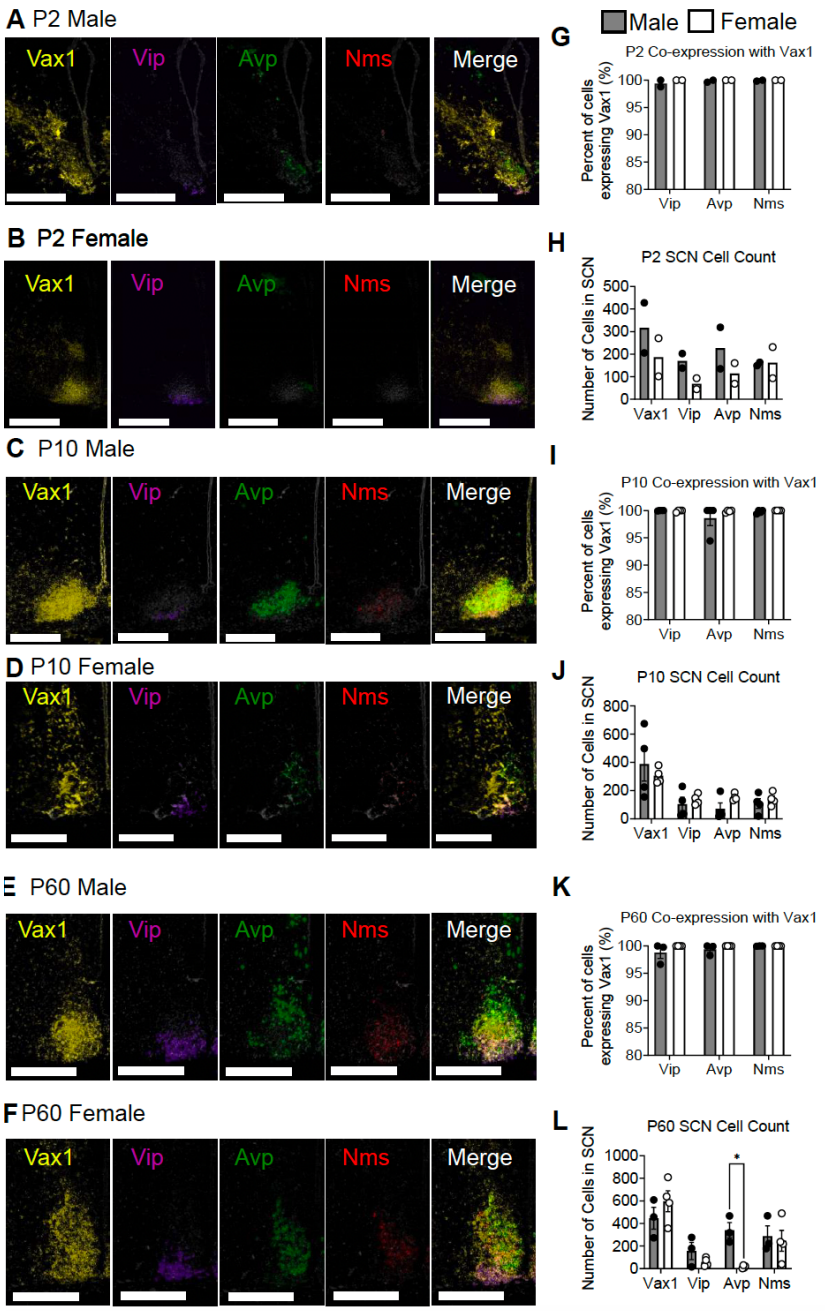

Figure 3.1. VAX1 is expressed in SCN VIP neurons from the early postnatal period through adulthood in both males and females. RNAscope® ISH representative images (A, B, C, D, E, F). Images at P2 are displayed with increased contrast, applied to all channels, to compare with P10 and P60. Percent co-expression between *Vax1* and *Vip*, *Avp,* and *Nms*-expressing neurons (G, I, K) and cell counts (H, J, L) in P2, P10 and P60 males and females at ZT5. Scale bar is 300 µm. Student's t-test *, $p < 0.05$.

As *Vax1* is ~100% co-expressed with *Vip* from P2 until P60 in males and females (Figure 3.1), we next generated a conditional knockout mouse to determine how loss of VAX1 in VIP neurons would impact SCN function. Using our RNAscope® ISH data from Figure 3.1, we first visually inspected all stained sections for potential *Vax1* expression in non-SCN *Vip* cells. The olfactory bulb is the only additional brain area that expresses *Vax1* which is also targeted by the Vip^{Cre} allele (Figure 3.2C). In the scenario the Vip^{Cre} allele does target some *Vax1* expressing cells in the olfactory bulb, a change in reproductive behavior could impact fertility data because olfaction is required for normal male reproductive behavior. To validate that the Vip^{Cre} allele targeted VAX1 expressing neurons of the SCN, we generated Vip:RosaTdTomato (Vip^{Cre}:Td) mice allowing identification of all neurons that are targeted by the Vip^{Cre} allele (Figure 3.2D, E). Using dual IHC, we quantified colocalization of VIP- and VAX1-expressing neurons in the SCN. Using tdTomato as a marker for VIP neurons in sections from Vip^{Cre}:Td animals, we found 38 ± 7% of SCN tdTomato+ neurons colocalized with VAX1 (n=4, 2 per sex, 36-101 neurons per animal). An average of 23 ± 5% of VAX1 neurons colocalized with tdTomato (n=4, 65-236 neurons per animal). This discrepancy between the RNAscope and dual IHC has numerous potential explanations, as detailed in the discussion.

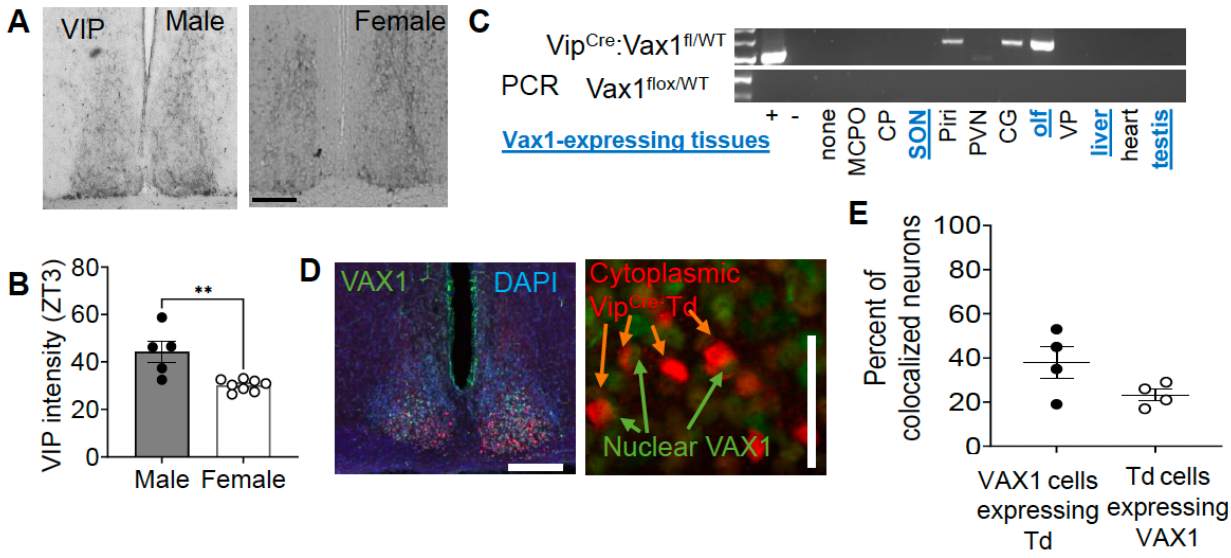

Figure 3.2. VIP peptide is sexually dimorphic in the SCN. A) Example image and B) quantification of SCN IHC for VIP in adult males and females at ZT3. Scale bar 100µm. Student's t-test, **, $p < 0.01$ C) RT-PCR shows Vip^{Cre} recombination of the $Vax1^{flox}$ allele in indicated tissues. Abbreviations stand for olfactory bulb (olf), magnocellular preoptic area (MCPO), caudoputamen (CP), supraoptic nucleus (SON), piraform area (Piri), paraventricular nucleus (PVN), cingulate gyrus (CG), and ventral palidum (VP). Blue, underlined text indicates tissues that are known to express *Vax1*. D) Example images and quantification E) of dual IHC for Vip^{Cre}:TdTomato (red, cytoplasm) and VAX1 (green, nuclear) expressing cells in the adult SCN (DAPI, nuclei). Scale 50 µm.

Conditional deletion of VAX1 in Vip^{Cre} neurons shortens SCN circadian period in females and males

To determine the role of VAX1 in VIP neuron circadian output, we evaluated wheel running behavior of Ctrl and $Vax1^{flox/flox}$:Vip^{Cre} ($Vax1^{Vip}$) mice in LD and constant darkness (DD). Wheel running patterns in LD of Ctrl and $Vax1^{Vip}$ females and males were comparable (Figure 3.3A-I, LD). Light is a strong entraining signal of the SCN, and activity rhythms in LD can mask weakened SCN function [202]. Following the initial LD period, mice were placed in DD for 28d to assess endogenous free-running period. Both male and female $Vax1^{Vip}$ mice showed a significantly shortened free-running period (Tau) compared to Ctrls (Figure 3.3E, two-way ANOVA, male $p = 0.0004$, female $p = <0.0001$) with no differences in Chi^2 amplitude (Qp). There were no changes in the number of wheel revolutions per day or activity duration (alpha, Figure 3.3F-I). To determine if the shortening of free-running period during DD resulted from a change in endogenous SCN circadian period, we generated triple transgenic mice crossing $Vax1^{Vip}$ mice with the PER2::LUC reporter mouse [46]. In agreement with the significantly shortened behavioral period of $Vax1^{Vip}$ males and females on DD, we found that the SCN of $Vax1^{Vip}$:PER2::LUC mice showed a significant shortening in period as compared to Ctrl males [Figure 3.4A, $t(27) = 2.936$, $p = 0.0067$] and females [Figure 3.4B, $t(24) = 2.125$, $p = 0.0440$]. No differences were found in the amplitude or phase relationships of PER2::LUC in the SCN of $Vax1^{Vip}$ males or females as compared to Ctrl, indicating that the rhythms in the SCN are not misaligned or significantly weakened (Figure 3.4C-F). Together these data show that loss of VAX1 in VIP neurons shortens SCN circadian output in both males and females.

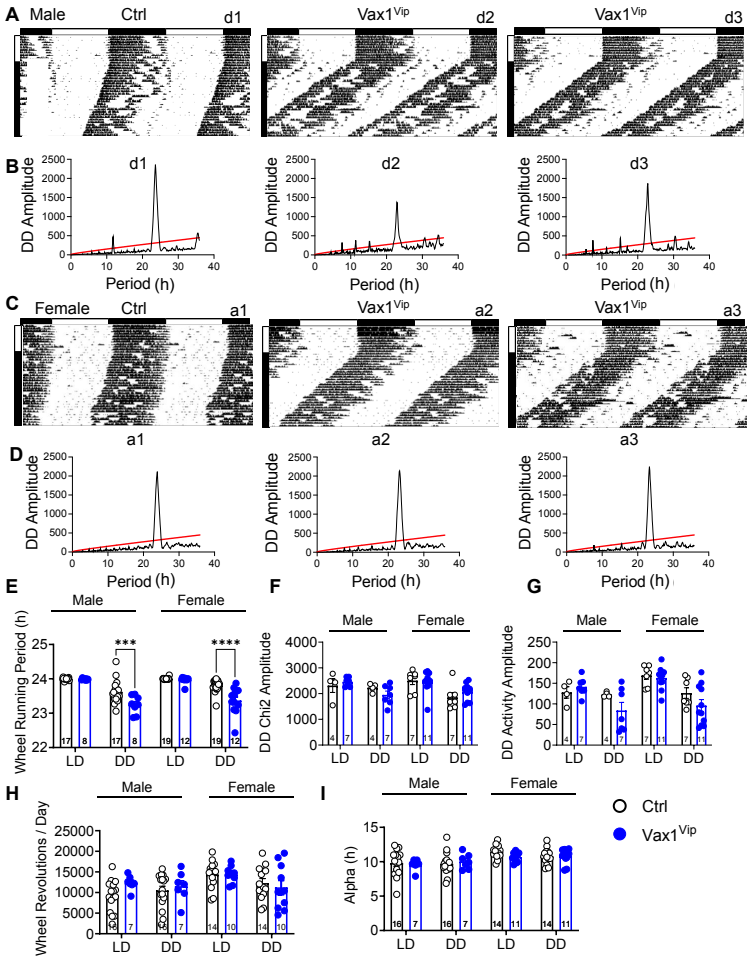

Figure 3.3. Vax1 deletion within VIP neurons shortens behavioral free-running period in males and females. A, B) Male and C-D) female Ctrl and Vax1^{Vip} mice were single housed with running wheels. A, C) Data show double plotted actogram activity with 14 days in LD12:12 (LD) followed by 28 days in constant darkness (DD). Data are presented in ClockLab normalized format. Horizontal bar above the actograms indicates lights on (white) and lights off (black) during the LD12:12 cycle. B, D) Chi^2 periodograms during 2 weeks in DD. Matching codes (a1, a2, etc.) on the upper right corner of each actogram and chi^2 periodogram indicate data from a particular mouse, with variable scaling indicated in the upper left. 14-day average wheel-running data were used for indicated analysis parameters in LD and DD. Average histogram data for E) Wheel-running period, F) Chi^2 amplitude, G) activity amplitude, H) Wheel revolutions, and I) activity duration (Alpha). Number within the bar indicates number of animals in each group. 3-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

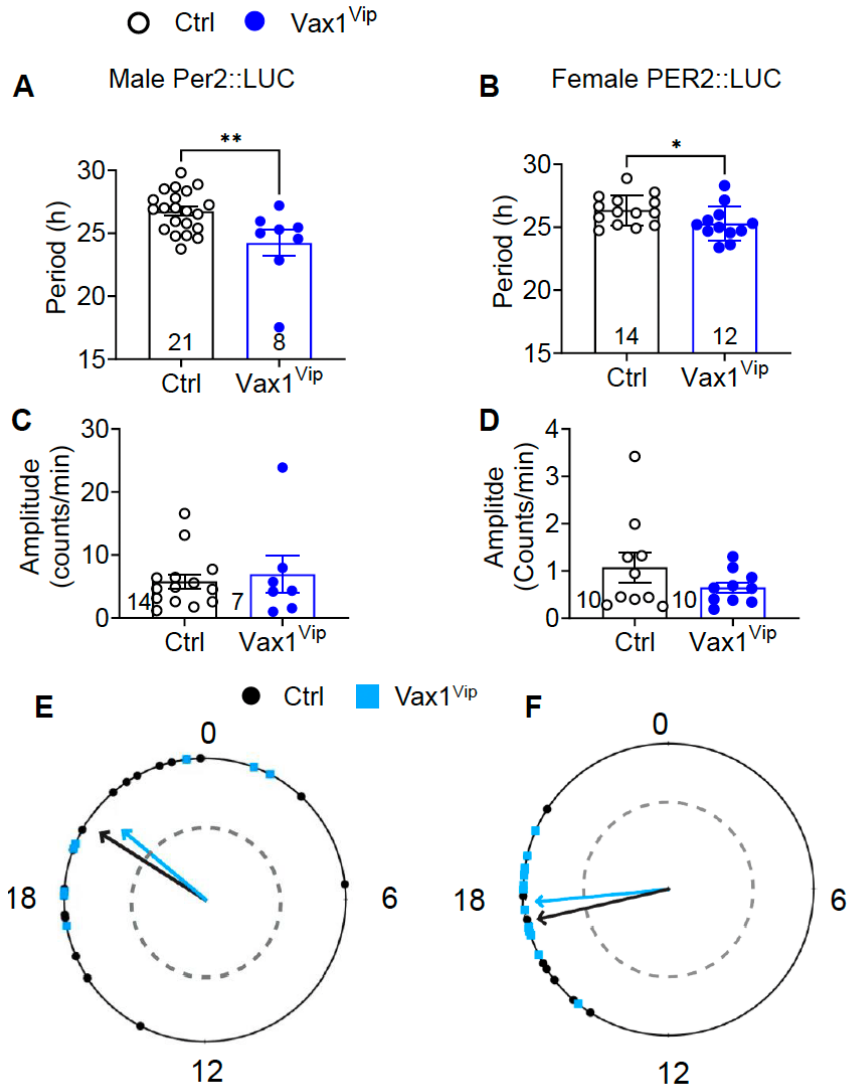

Figure 3.4. Vax1^{Vip} SCN has a shortened PER2::LUC period in *ex vivo* culture. Histogram of PER2::LUC SCN A, B) circadian period and C, D) amplitude from control and Vax1^{Vip}:PER2::LUC females (combined proestrus and diestrus) and males. Statistical analysis by Student's t-test, *, $p<0.05$ **, $p < 0.01$, n = 7-16. E) PER2::LUC phase (time of first peak) in the SCN of control and Vax1^{Vip}:PER2::LUC males and F) females, n = 7-16. Mean times of first peak are indicated by vector lines, and symbols indicate individual data points. Data were analyzed via the Rayleigh Test of Uniformity, where crossing the dotted gray line indicates significant clustering ($p < 0.05$), and the Watson's Two-Sample Test of Homogeneity. No significant differences were found in females between estrous stages.

VAX1 regulates molecular clock gene expression in VIP neurons through a direct mechanism

A shortened SCN period can be driven by both changes in SCN peptide expression and changes in molecular clock gene expression. We have previously shown that VAX1 promotes Per2-luciferase plasmid expression using transient transfection assays [121]. However, we have not yet demonstrated whether this action is direct or indirect. To assess if VAX1 directly binds with our top candidate ATTA site of the mouse *Per2* DNA regulatory region [121], we used EMSA. We found that VAX1 directly binds the ATTA site at +1774/1770bp from the transcriptional start site of the mouse *Per2* gene (Figure 3.5A, Super shift is indicated by *). In addition to ATTA sites in the *Per2* regulatory region, the *Bmal1* regulatory region also contains numerous ATTA sites. Using transient transfections, we found that VAX1 promotes *Bmal1*-luciferase expression (Figure 3.5B). Site-directed mutagenesis of ATTA-like sites in the *Bmal1* regulatory region (Table 3.1) showed a modest increase in fold-change of VAX1-driven *Bmal1*-luciferase expression in transfected cells (Figure 3.5B). Interestingly, VAX1 can directly bind to all the identified ATTA-sites tested by EMSA (EMSA, Figure 3.5C, supershift indicated by *). This identifies for the first time that VAX1 can directly bind to the regulatory regions of *Per2* and *Bmal1* and provides a mechanism by which changes in VAX1 expression can directly impact molecular clock function. Next, to determine if loss of VAX1 in VIP neurons would significantly impact *Bmal1* expression in *Vip* neurons, we performed RNAscope® for *Bmal1, Vip,* and *Avp* in the adult SCN of Ctrl and $Vax1^{Vip}$ females (Figure 3.6). Note these experiments were completed in proestrus at ZT16, with the goal to have the most hormonally challenging environment in the female body present at the time of sample collection. We found that in Ctrl and $Vax1^{Vip}$ females almost 100% of *Vip* and *Avp* neurons co-expressed *Bmal1* (Figure 3.6A, B). Despite a trend in a reduction in *Vip* in the SCN of $Vax1^{Vip}$ females (Figure 3.6C), as well as a trend in the reduction in cells co-expressing *Vip* and *Bmal1* (Figure 3.6D), no significant difference in any of the studied transcripts, or colocalization of transcripts were identified (Figure 3.6C, D).

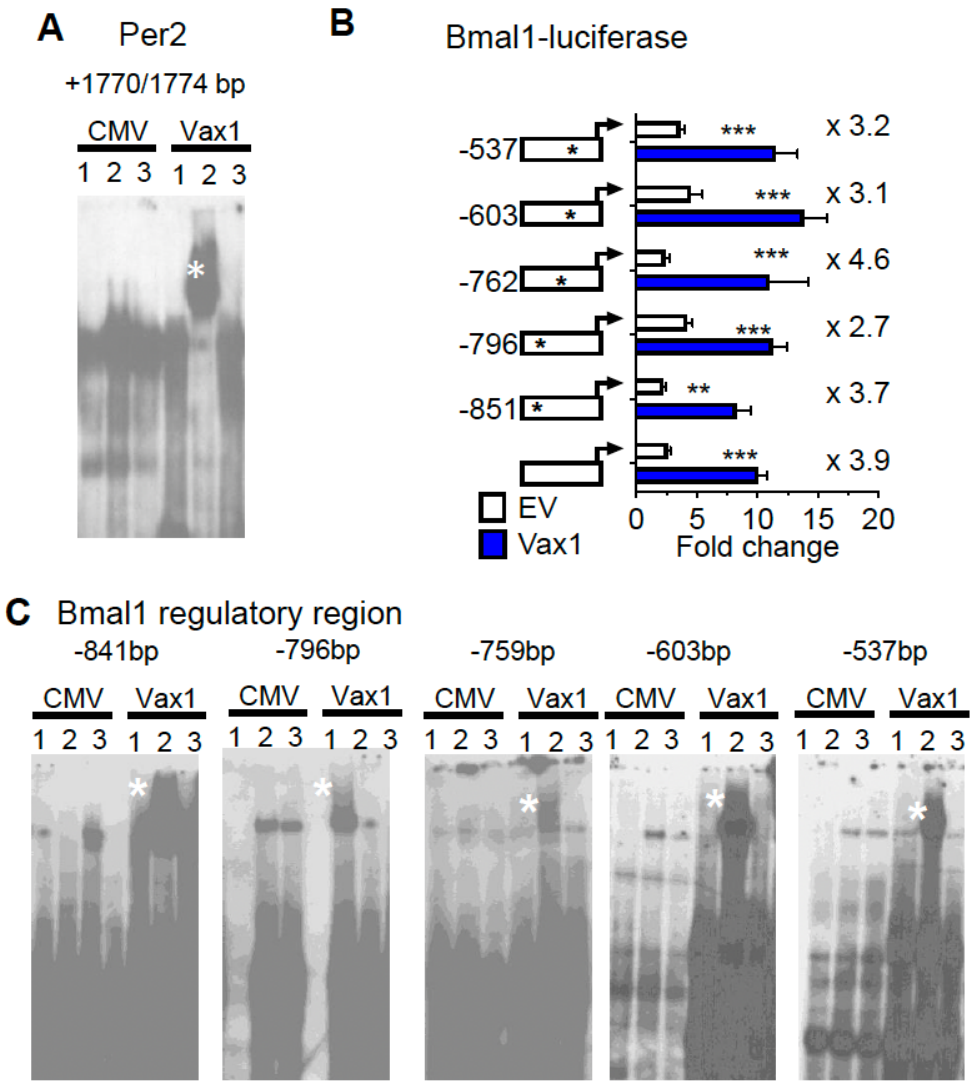

Figure 3.5. VAX1 binds directly to regulatory regions of molecular clock genes *Per2* and *Bmal1*. A, C) EMSA assay of COS-1 cells, represents in Lane 1: pCMV-Flag (CMV, empty vector), Lane 2: Vax1-Flag plasmid and Lane 3: Vax1-Flag plasmid + anti Flag antibody. White star indicates super shift. Example gels of n = 3. B) Transient transfections of NIH3T3 cells with the mouse *Bmal1* regulatory region driving luciferase (Bmal1-luciferase) with and without Vax1 overexpression vector (20 ng) or its empty vector (EV, pCMV6, 20 ng). Numbers indicated with the stars on the regulatory regions refer to ATTA sites that have been mutated (see Table 1). Statistical analysis by Two-way ANOVA mixed effect model, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, n = 4-6 in duplicate or triplicate.

Table 3.1. Primers used for site-directed mutagenesis in the *Bmal1* regulatory region. Primers used to mutate ATTA sites to GCCG within the *Bmal1* promoter plasmid. Position refers to the number of base pairs from the transcription start site. Underlined sequences indicate mutated bases. All primers were designed using NEBase Changer.

Position	Sequence
-841	TGTCCATAACATGTAATAGAATCTTGCTCA
-796	CTCAGTACTCGCGATTATGCCCCTGCCTCA
-759	CTTGAGGGTTGGAATTACAGACTACGCCAC
-603	AAATGCGCTGGCTATTAGCGCTGTGGTTCC
-537	CACTCTGTGTTCCTAATATGTGGTTTCCTA

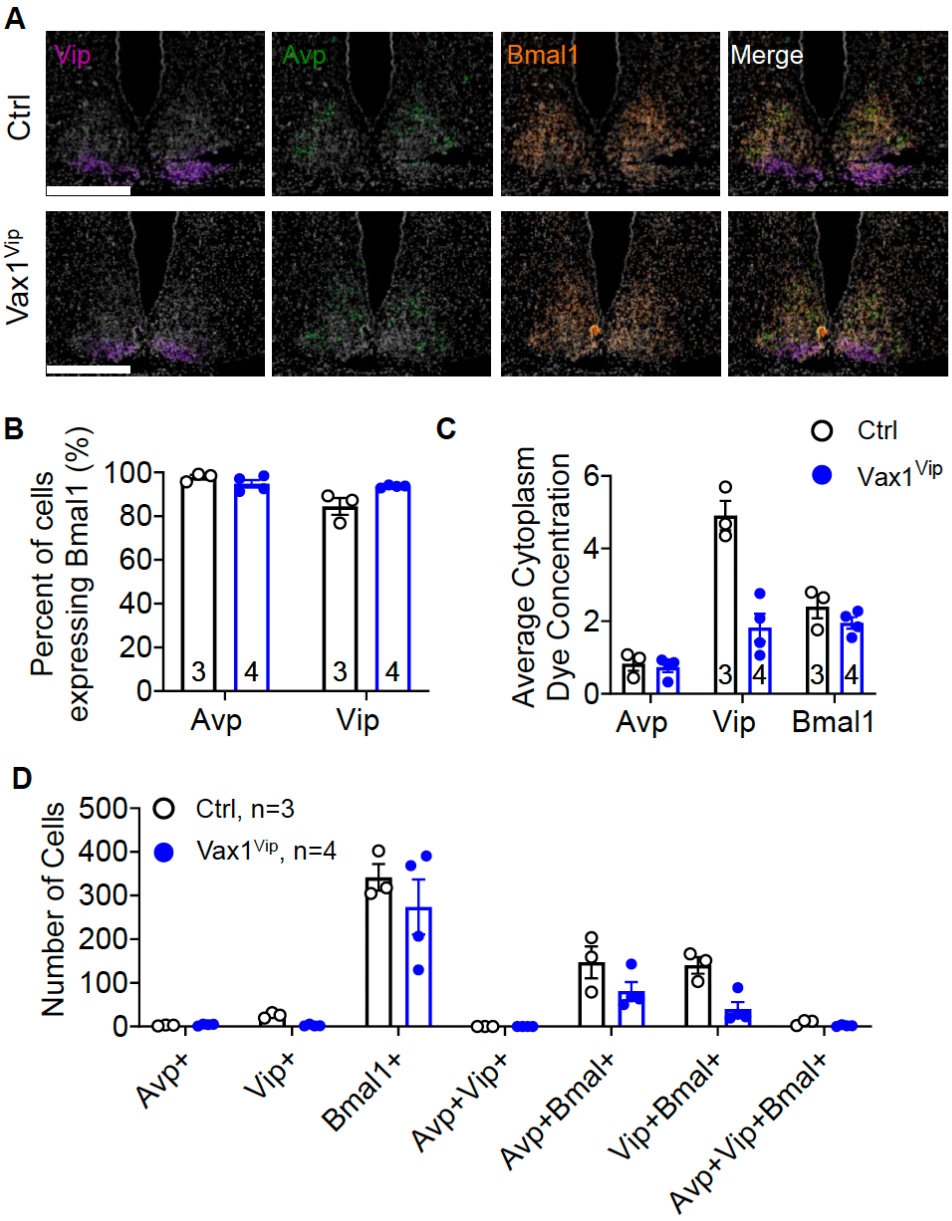

Figure 3.6. Loss of Vax1 in VIP neurons does not reduce *Vip* and *Bmal1* expression in the SCN. RNAscope® assay at ZT16 in the SCN of proestrus Ctrl and Vax1^{Vip} females. A) Example images of RNAscope® assay for *Vip* (blue), *Avp* (red) and *Bmal1* (green). N = 3-4 per group. Scale bar 300 µm. B) Percentage of cells that co-express *Bmal1* with *Avp* or *Vip*. Mann-Whitney, $p > 0.05$. C) Average cytoplasm dye concentration reflecting mRNA transcripts for *Avp*, *Vip*, and *Bmal1* Mann-Whitney, $p > 0.05$. D) Number of cells expressing indicated combinations of *Avp*, *Vip*, and *Bmal1*. Mann-Whitney, $p > 0.05$.

Vax1^{Vip} females have lengthened estrous cycles and deregulated sex steroids, whereas males have increased sperm count

The SCN provides daily neuronal and hormonal signals aligning circadian timekeeping in peripheral reproductive tissues allowing coordination between hormone release and increased tissue sensitivity improving reproductive function [58], [121], [152], [155]. To determine if Vax1^{Vip} mice have misaligned circadian phase of their reproductive tissues, we recorded PER2::LUC expression in the pituitary, ovary (female), uterus (female) and epididymis (male) of triple transgenic mice. No tissues were found to have significant differences in period (Table 3.2), amplitude (Table 3.2), or time of first PER2::LUC peak (Table 3.2, phase). These data indicate that the weakened SCN output of Vax1^{Vip} mice does not significantly impact circadian timekeeping in the studied peripheral tissues but does not preclude disruptions to fertility. To determine if reproductive function is impacted in Vax1^{Vip} mice, we first evaluated pubertal onset. At pubertal onset body weight was comparable between Ctrls and Vax1^{Vip} in both sexes (Table 3.3). Male pubertal onset, as evaluated by preputial separation (PPS) was slightly delayed [Figure 3.7A, $t(18) = 2.211$, $p = 0.040$], whereas female pubertal onset, as assessed through vaginal opening (VO) and first estrous, were comparable between Ctrls and Vax1^{Vip} females (Table 3.3). There was no impact on male reproductive function (Table 3.3) apart from significantly increased total sperm count [Figure 3.7B, $t(12) = 3.101$, $p = 0.009$]. Vax1^{Vip} males had normal testis size and percent motile sperm (Table 3.3). The increase in total sperm pool was not associated with changes in basal LH and FSH levels in males (Table 3.3). Both Vax1^{Vip} males and females were comparable to Ctrls for the number of litters generated in 90 days, days to first litter, and litter sizes (Table 3.3). Despite the normal fertility in females, Vax1^{Vip} females had a significant lengthening of the estrous cycle [Figure 3.7C, $t(19) = 2.307$, $p = 0.033$] with a similar amount of time spent in each cycle stage as compared to Ctrls [Two-way ANOVA, $F(1, 10) = 2.500$, $P=0.1449$]. This lengthening in estrous cycles was associated with a reduction in FSH, estrogen, and ovarian weight in Vax1^{Vip} females (Figure 3.7D, E, F), but did not impact basal LH or uterine weight in diestrus (Table 3.3).

Table 3.2. Summarized data of male and female Vax1^{Vip} PER2::LUC recordings. PER2::LUC lumicycle data from male and proestrus female tissues. Phase data were analyzed using a Rayleigh test followed by a Watson two-sample test of homogeneity.

	Ctrl (Avg±SEM)	Vax1^{Vip} (Avg±SEM)	Student's t-test, P
Ovary Period (h)	25.6 ± 0.3	25.2 ± 1.5	n = 3-6, P = 0.87
Ovary Amplitude (counts/min)	27.4 ± 11.2	8.7 ± 2.6	n = 3-6, P = 0.40
Uterus Period (h)	25.7 ± 0.5	27.2 ± 1.7	n = 3-6, P = 0.25
Uterus Amplitude (counts/min)	43.3 ± 9.6	9.1 ± 7.2	n = 3-6, P = 0.10
Female Pituitary Period (h)	24.2 ± 0.2	25.5 ± 0.3	n = 3-6, P = 0.18
Female Pituitary Amplitude (counts/min)	10.5 ± 3.3	15.4 ± 11.5	n = 3-5, P = 0.58
Female Arcuate Period (h)	25.3 ± 0.7	24.1 ± 0.6	n = 3-5, P = 0.39
Female Arcuate Amplitude (counts/min)	1.1 ± 0.4	1.3 ± 0.2	n = 3-5, P = 0.77
Epididymis Period (h)	24.5 ± 0.2	25.5 ± 0.5	n = 8-9, P = 0.12
Epididymis Amplitude (counts/min)	12.9 ± 4.9	12.7 ± 1.0	n = 8, P = 0.98
Epididymis Phase (h)	4.5 ± 0.1	4.1 ± 0.1	n = 8-9 , P = 0.81
Male Pituitary Period (h)	25.3 ± 0.3	24.9 ± 0.6	n = 8-23, P = 0.447
Male Pituitary Amplitude (counts/min)	16.5 ± 2.5	9.8 ± 2.6	n = 8-20, P = 0.14
Male Arcuate Period (h)	24.7 ± 0.5	24.9 ± 0.4	n = 8-22, P = 0.84
Male Arcuate Amplitude (counts/min)	1.9 ± 0.4	1.8 ± 0.6	n = 8-22, P = 0.88

Table 3.3. Fertility parameters in Vax1^{Vip} mice. Pubertal onset was evaluated by vaginal opening (VO) in females and preputial separation (PPS) in males. Gonadal, uterine, and circulating hormone values are from adult Vax1^{Vip} males and diestrus/metestrus females. Statistical analysis by Student's t-test.

	Ctrl (Avg±SEM)	Vax1^{Vip} (Avg±SEM)	Student's t-test, P
Age at VO (days)	28.7 ± 0.5	29.1 ± 0.9	n = 10-26, P = 0.67
Weight at VO (g)	12.0 ± 0.2	12.5 ± 0.5	n = 10-26, P = 0.36
Weight at PPS (g)	13.1 ± 0.4	14.0 ± 0.7	n = 8-12, P = 0.22
Diestrus uterus weight (mg)	71.6 ± 9.5	71.5 ± 7.5	n = 8-16, P = 0.99
Age at first estrus (days)	34.7 ± 0.9	34.0 ± 1.4	n = 7-22, P = 0.70
Testis weight (mg)	103.8 ± 4.4	100.4 ± 3.5	n = 4-6, P = 0.59
LH (ng/ml) diestrus female	0.51 ± 0.13	0.17 ± 0.04	n = 7-14, P = 0.09
LH (ng/ml) male	0.35 ± 0.08	0.40 ± 0.19	n = 10-14, P = 0.83
FSH (ng/ml) male	12.58 ± 1.13	11.41 ± 1.12	n = 12-14, P = 0.47
Percent Motile Sperm	35.09 ± 2.41	31.21 ± 4.58	n = 3-9, P = 0.70
Female Litter Size	7.14 ± 1.55	8.34 ± 0.84	n= 6-22, P = 0.16
Male Litter Size	7.14 ± 1.55	7.48 ± 1.52	n= 10-22, P = 0.79
Female Litters in 90 days	2.05 ± 0.65	2.33 ± 0.82	n= 6-22, P =0.61
Male Litters in 90 days	2.05 ± 0.65	2.43 ± 0.79	n= 7-22, P = 0.38
Female Days to first litter	25.69 ± 7.86	21.33 ± 1.51	n= 6-16, P = 0.31
Males Days to first litter	25.69 ± 7.86	26.64 ± 6.05	n= 11-16, P = 0.91

Reduced VIP peptide in the SCN of Vax1^{Vip} mice is associated with increased GnRH neuron sensitivity to kisspeptin in females, but not in males

VIP neurons from the SCN project directly to GnRH neurons and indirectly through AVP to kisspeptin neurons. The anterior pituitary releases LH into the circulation upon GnRH release at the median eminence, allowing an indirect approach to study GnRH neuron function. To determine if the reduction in VIP peptide in the Vax1^{Vip} SCN impacted GnRH neuron response to kisspeptin, we performed hormone challenges in mice. We first confirmed that the pituitary responded to GnRH by increasing LH release through an i.p. injection of GnRH. As expected, the fold change in LH in response to a GnRH challenge was comparable between Ctrl (9.07 ± 1.97, n = 4) and Vax1^{Vip} males [12.09 ± 2.43, n = 6, t(8) = 0.885, p= 0.401], and between Ctrl (10.50 ± 3.37, n=8), and Vax1^{Vip} females [6.95 ± 3.84, n = 4, t(10) = 0.641, p= 0.535]. To assess if the GnRH neuron response to kisspeptin was impacted in Vax1^{Vip} males and females, we next performed an i.p. kisspeptin challenge. There were no differences between LH release in Vax1^{Vip} (5798 pg/mL ± 583, n = 4) males and Ctrls [5325 pg/mL ± 670, n = 5, t(7) = 1.11, p = 0.303]. In contrast, Vax1^{Vip} females had an increased release of LH in response to kisspeptin at 5 and 10 minutes as compared to Ctrls (Figure 3.7G, mixed-effects analysis, 5 minutes p = 0.0102, 10 minutes p = 0.0257), as well as an overall increase in LH release [Figure 3.7H, t(7) = 2.560, p = 0.0376]. Such alteration in the neuroendocrine network regulating LH release would be expected to impact female estrous cyclicity, which relies on precisely timed hormone release and sex-steroid-feedback.

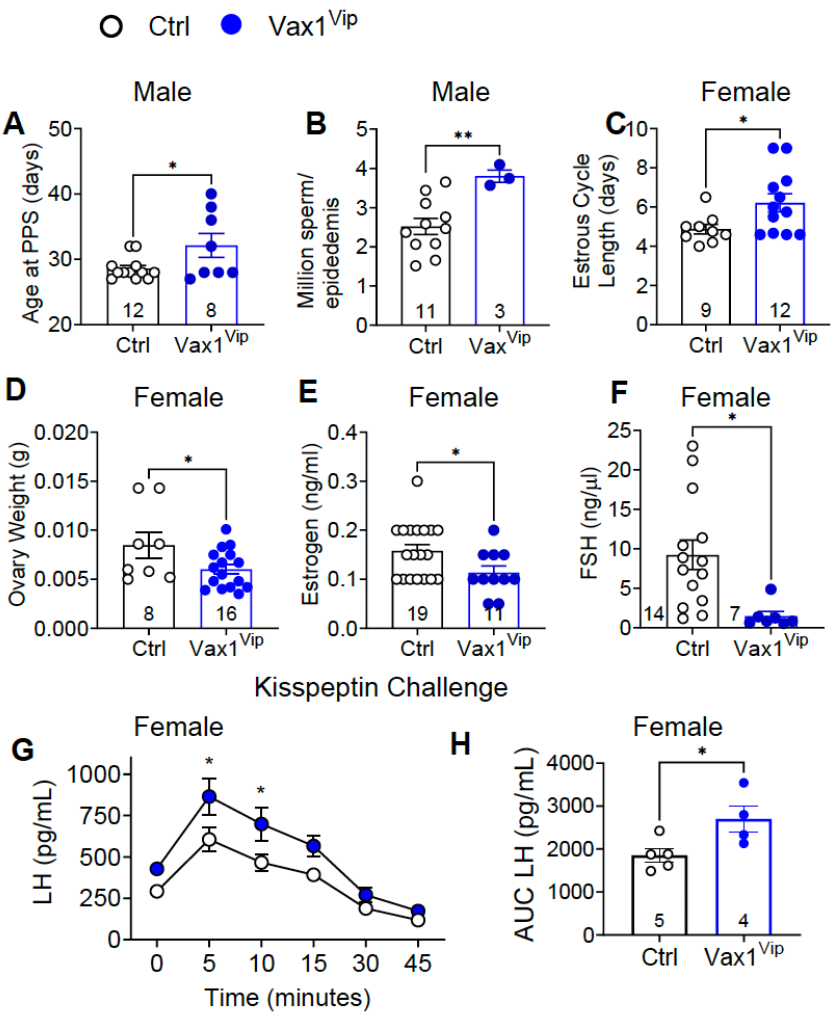

Figure 3.7. Vax1^{Vip} females have a reduction in ovary weight, estrogen, and FSH, as well as an increased sensitivity to kisspeptin. A) Age at preputial separation (PPS) and B) million sperm per epididymis in Ctrl and Vax1^{Vip} males, n indicated in graphs, Student's t-test, *, p<0.05; **, p <0.01. C) Estrous cycles were evaluated in females, and average estrous cycle length was established. Student's t-test, *, p < 0.05. D) Ovary weight, E) Circulating estrogen and F) Circulating FSH of diestrus females. n indicated in graphs, Student's t-test, *, p < 0.05. G) Circulating LH levels in diestrus females evaluated over a 45-minute time period in response to an i.p. kisspeptin injection. Mixed Effects analysis, *, p < 0.05. and H) the resulting area under the curve. Student's t-test, *, p < 0.05.

Hypothalamic imbalance between *Vip* and *Avp* in the Vax1^{Vip} hypothalamus is associated with increased basal corticosterone and depressive-like symptoms in females

AVP is expressed outside the SCN and is highly expressed in the paraventricular nucleus (PVN), a direct target of SCN neurons. The PVN is a well-established relay site playing a significant role in several autonomic functions, including stress [203]–[205]. To determine how *Avp* levels were impacted in the hypothalamus of Vax1^{Vip} females, we analyzed *Avp* using RNAscope® assay. Interestingly, we found that Vax1^{Vip} females, which have comparable *Avp* mRNA in the SCN to Ctrl (Figure 3.1F, L), display a significant increase in *Avp* transcript and cell numbers in the PVN (Figure 3.8A-C). This increase of *Avp* in the PVN of Vax1^{Vip} females provides a potential link to activation of the stress axis. In agreement with this, basal corticosterone levels were overall increased at ZT3 in Vax1^{Vip} mice [Figure 3.8D, two-way ANOVA, $F(1,12) = 5.868$, $p = 0.032$]. Finally, to determine if these known risk factors for depression would reflect an increase in depressive-like behavior in Vax1^{Vip} mice, we tested males and females in the Porsolt forced swim test. We found that Vax1^{Vip} females (metestrus, ZT13) exhibited increased depressive-like behaviors as shown by an increase in the percentage of time floating (Figure 3.8E) as compared to Ctrl females [$t(17) = 2.121$, $p = 0.0489$], while Vax1^{Vip} males were comparable to Ctrls [$t(11) = 0.5889$, $p = 0.5678$]. The increased time floating in the Porsolt forced swim tests of the Vax^{Vip} females correlated with increased circulating corticosterone 1 h after the swim test [Figure 3.8F, $t(11) = 3.595$, $p = 0.0042$].

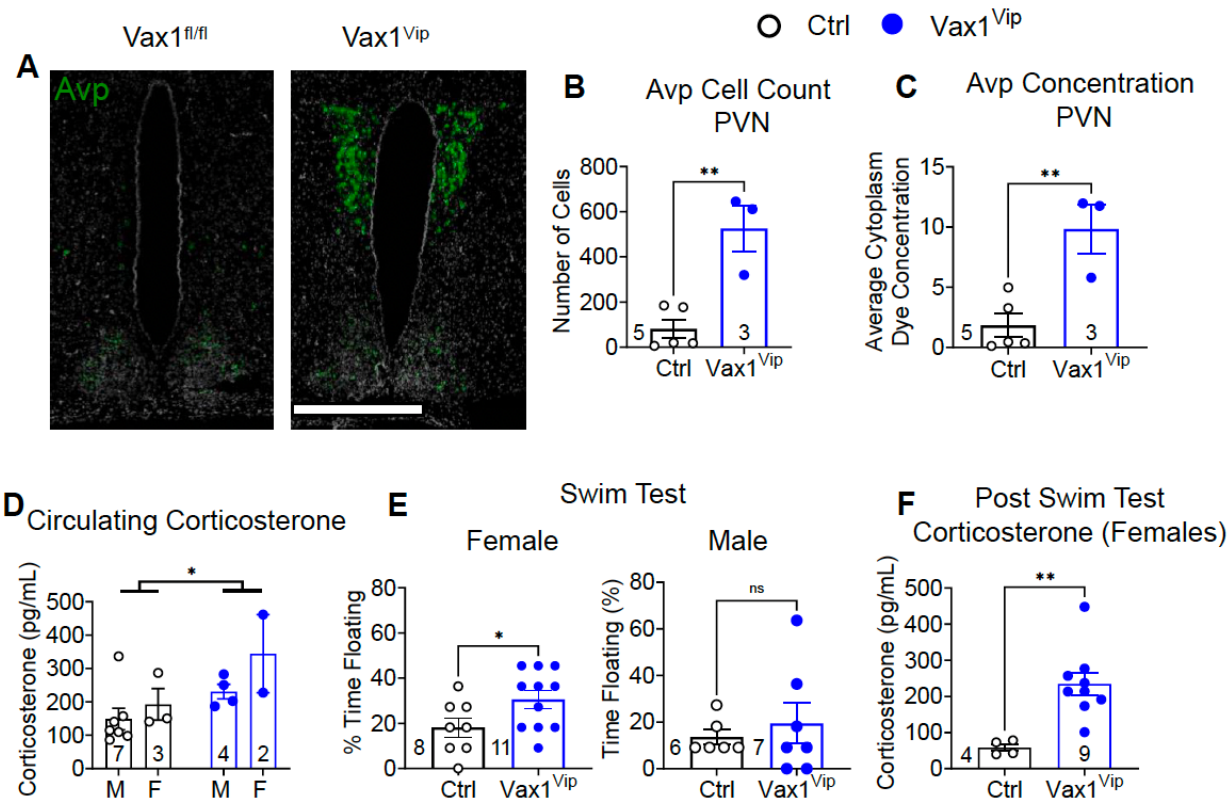

Figure 3.8. Vax1^{Vip} females have increased AVP cell count and concentration in the PVN, increased corticosterone, and increased depressive-like behaviors. A) Example images of RNAscope® detection for *Avp* in the SCN and paraventricular nucleus (PVN) of proestrus females at ZT16. Scale bar is 600 µm, n=3-5. Quantification of RNAscope® assay by B) cell counts and C) dye concentration of *Avp* in the PVN, n=3-5, Student's t-test, **, p <0.01. D) Corticosterone was measured at ZT3-5 in males (M) and females (F), Two-way ANOVA, *, p<0.05. E) To test depressive-like behavior, metestrus female or male mice were tested at ZT13 by a Porsolt forced swim test, and the % time floating assessed. N indicated in graph, Student's t-test, *, p<0.05. F) Corticosterone levels in circulating blood 1 h following the Porsult forced swim test. n = 4-9, Student's t-test, **, p<0.01.

Discussion

This work explores VIP neurons within the SCN as a neurological point of overlap between circadian disruption, reproduction, and depressive-like behaviors. Here, we leverage the highly localized co-expression between the homeodomain transcription factor VAX1 in VIP neurons of the SCN to develop a conditional knockout mouse model that exhibits abnormal circadian timekeeping, reproductive axis function, and mood. Excitingly, this novel mouse model suggests that VIP neurons might provide a shared neurological underpinning between reproductive and mood disorders.

Vax1 is co-expressed in *Vip, Nms,* and *Avp* neurons from development to adulthood and regulates molecular clock gene expression

The SCN retains a high expression of numerous homeodomain transcription factors after development [120], [125], [131], [146], [206], including *Vax1*. *Vax1* is critical in brain and neuronal development [14], [120], [121], [199], [207] and highly expressed in the developing mouse brain before it becomes refined to the adult hypothalamus, primarily in the SCN, during the early postnatal period [49], [121]. We have previously shown that conditional deletion of *Vax1* in late neuronal development using the Synapsin^{Cre} allele reduced VIP expression in the adult SCN [121], but this previous study did not address how many VIP neurons co-expressed *Vax1*. Here we find that ~100% of *Vip*-expressing neurons co-express *Vax1* at P2 and maintain a close to 100% co-expression at P10 and P60. In addition, we show that *Vax1* is also co-expressed by ~100% of *Nms* and *Avp* neurons at P2, P10, and P60. This shows for the first time that *Vax1* is highly expressed in three primary SCN neuron populations from the early postnatal period into adulthood, suggesting a role of VAX1 in regulating the function of these neuronal populations. The close to 100% co-expression of *Vax1* and *Vip* strengthens the value of our conditional knock-out model as a novel tool to specifically study SCN VIP neurons. This high level of overlap, coupled with the primary restriction of *Vax1* expression within VIP neurons in adulthood to the SCN [49], [121] allows for targeted impairment of VIP neurons within the SCN alone. This approach allows us to build upon previous work that identified the importance of VIP in circadian and reproductive function using VIP knockout mice [76], while avoiding some pitfalls of other methods of selectively targeting VIP neurons within the SCN, such as damage to

other neurons or brain nuclei via surgical methods due to the ventral location of the SCN [81], [208]. One concern with conditional knockout mice is that off-target recombination may impact other brain or peripheral functions or have a negative impact on development. In our model, we found Vip^{Cre}-mediated recombination outside of the SCN was restricted to the olfactory bulb, which also expresses *Vax1*. However, as a functioning olfactory bulb is pivotal to male mating [209] and our male mice bred normally, it is likely that the olfactory bulb is not significantly affected in this model. We also generated Vip^{Cre}:Td mice to identify neurons that were targeted by the Vip^{Cre} allele prior to tissue collection to validate that the Vip^{Cre} allele specifically targeted VAX1 expressing neurons of the SCN. We confirmed that SCN tdTomato+ neurons colocalized with VAX1, and VAX1 neurons colocalized with tdTomato, although these percentages were lower than predicted by RNAScope® ISH assay. The differences in co-localization between mRNA and protein results could reflect different sensitivity limits between the visualization approaches, or alternatively may provide evidence that not all *Vax1* mRNA is being translated into protein. Another possibility is that VAX1 expression could be circadian at the mRNA and/or protein level, leading to differences in expression that are time-of-day dependent, although future studies will be needed to determine this.

As a transcription factor that binds to ATTA and ATTA-like sites, a common sequence in the DNA, VAX1 has a high number of genes it can potentially regulate. Here, we build upon our previous work that determined the ability of VAX1 to promote Per2-luciferase expression [121] by providing evidence that this occurs via direct binding of VAX1 to regulatory regions of *Per2*. In addition to *Per2*, another core-component of the molecular clock, *Bmal1* also contains numerous ATTA-like sites in its regulatory region. We found here that VAX1 can directly bind to all the identified *Bmal1* ATTA-sites tested by EMSA, in addition to promoting Bmal1-luciferase expression. This work provides a mechanism by which changes in VAX1 expression can directly impact molecular clock function by VAX1 binding to the regulatory regions of *Per2* and *Bmal1*. Excitingly, $Vax1^{Vip}$ mice presented with a shortening in SCN PER2::LUC period in *ex vivo* recordings, as well a shortened free-running period, together supporting a role of VAX1 as a novel regulator of both molecular clock expression and function. Future work will be required to determine if loss

of VAX1 in VIP neurons causes a circadian phase shift in VIP neurons, and/or if a loss of VAX1 leads to a reduction in molecular clock transcript expression, which could impact clock-controlled gene expression and phase.

Circadian timekeeping is impaired in $Vax1^{Vip}$ mice, where differences in SCN peptide transcripts levels may underlie sex-specific vulnerability to circadian disruption

VIP neurons within the SCN are an important coordinator of the circadian timekeeping system [186]. Given this, it is no surprise that the $Vax1^{Vip}$ mouse model demonstrates altered SCN output, as indicated by the shortened $Vax1^{Vip}$ free-running period in both sexes. This finding is consistent with work done by others indicating that a decrease in VIP within the SCN results in a shortened free-running period [76]. However, VIP rarely acts alone in the regulation of circadian behaviors, and other peptides and components of the molecular clock exert strong influences on locomotor period [70]. A shortened free-running period can be caused by an increase in SCN AVP [210]–[212] or reductions in *Bmal1* expression [213]. Others have found that *Bmal1* is expressed rhythmically in *Avp*- and *Vip*-expressing neurons [67] and that deletion of *Bmal1* from *Avp*-expressing neurons can lengthen free-running periods in mice [144]. As $Vax1^{Vip}$ females do not show significant changes in *Avp* in the SCN, and only trended towards decreased *Vip* expression, in addition to a non-significant reduction in neurons co-expressing *Bmal1* and *Vip*, the cause of the shortened period in $Vax1^{Vip}$ mice remains unknown. Future work will aim at determining if these trending reductions in *Vip,* combined with a trend in reduced *Bmal1* expression in *Vip* neurons together might contribute to the shortened SCN period of $Vax1^{Vip}$ mice.

Sex differences are well-documented in SCN morphology and cellular function, previously reviewed in [214], [215]. There is strong evidence that VIP is sexually dimorphic, with increased VIP expression in human males [216], [217], as well as increased *Vip* transcript in male nocturnal laboratory rats [218] and diurnal Nile Grass rats [219]. Our data support and extend these findings, where Ctrl male mice also exhibit increased *Vip* transcript and VIP compared to females. A potential mechanism guiding this sex difference could rely on the influence of the gonadal hormones estradiol and testosterone, which modulate VIP expression in the SCN [218]–[221]. Our data, and others, suggest that sex differences in both

Vip transcript and peptide levels [214] occur post-puberty. Recent work has demonstrated detailed spatial patterning of the onset and development of *Avp* and *Vip* transcription [222]. Our data support the conclusion that SCN *Vip* neuron development is not complete at P10; however, we find decreased *Vip* transcript and protein in the adult female SCN compared to males, while increased *Vip-tdT*+ cell numbers were found in a lateral cluster of the SCN [222]. There are several potential rationales for these differences, including delays between transcription onset and tdT-labeling and low *Vip-tdT*+ co-expression with VIP-IHC (less than 30%) that may lead to different results from our mRNA transcript measure. Nevertheless, these neuropeptide sex-differences highlight the need for continued investigation in both males and females to further our understanding into how SCN function drives circadian output in both sexes and the role of sex steroids therein.

Vax1^{Vip} males exhibit normal fertility, while females display modest dysregulation of the reproductive axis

Circadian timekeeping is essential for coordinating hormone release and increased tissue sensitivity within reproductive tissues [58], [121], [155]. The SCN modulates the timing of hormone release through direct and indirect projections to GnRH neurons, which in turn regulate the release of LH and FSH [223]–[225]. These hormones are required for reproductive health through the production of testosterone and spermiogenesis in males and ovulation, embryo implantation, and follicular health in females [226]–[228]. Though more frequently studied in females, current studies suggest that severe circadian disruption, through changes either in light exposure or via direct disruption to the SCN, may have a mild influence on male fertility [229]. Interestingly, we found that Vax1^{Vip} males had delayed pubertal onset. Given the importance of both SCN *Avp* and *Vip* in LH release [83], [183], [230], a required hormone for pubertal onset [231], it will be of interest for future studies to examine SCN neuropeptide expression in Vax1^{Vip} males undergoing puberty. Interestingly, aside from delayed pubertal onset, there were no significant deficits in Vax1^{Vip} male reproductive function, and Vax1^{Vip} males displayed an increase in total sperm through an unknown mechanism. These data support a theory that a less stringent circadian control may favor male fertility. Together our data, coupled with evidence from genetic knockout and light-disruption studies [61], [229], [232], indicate that male fertility is resilient when faced with circadian challenges.

In contrast to males, circadian disruption and impaired SCN function are known to have a strong impact on female reproduction [6], [22], [155], [233]. VIP, in particular, has an important role in the female reproductive axis, where VIP knockout females have lengthened estrous cycles and are sub-fertile, resulting in fewer liters of smaller sizes [77]. It is likely that some of the sub-fertility in full body VIP knockout females is driven by VIP neurons within the SCN, as our Vax1^{Vip} females also displayed lengthened estrous cycles. Our data are further supported by work in progress, showing that surgically ablated VIP neurons within the SCN also lengthened estrous cycles [87]. Although Vax1^{Vip} estrous cycles were lengthened, we did not see changes in litter sizes or number of litters that are associated with full VIP knockout females. Taken together, these data indicate estrous cycle length is influenced by Vip-expressing neurons within the SCN.

The estrous cycle is regulated by hormonal feedback throughout the reproductive axis. Within the hypothalamus, VIP neurons directly project onto GnRH neurons to regulate the frequency of GnRH release to the pituitary [180] and indirectly through projections onto AVP neurons in the anteroventral paraventricular nucleus to regulate kisspeptin neurons that modulate the surge release of GnRH needed for ovulation [234], [235]. Although only a single dose of kisspeptin was tested, we found that Vax1^{Vip} females had a greater release of LH in response to a kisspeptin challenge than Ctrls, a difference we did not observe in the males. The normal pituitary response to GnRH and normal circadian rhythms in *ex vivo* pituitary explants of Vax1^{Vip} females, combined with the absence of *Vax1* in gonadotropes as shown by qPCR in isolated gonadotrope cells from female mice [199], [236] and single cell RNAseq [personal communication, [236], [237]], suggest it is unlikely that the increased LH release in response to kisspeptin is associated with abnormal gonadotrope function. One possibility for the sex difference in kisspeptin-induced changes in LH release in Vax1^{Vip} mice might be linked to differences in the neuronal circuit encompassing the sexually dimorphic anteroventral periventricular nucleus kisspeptin neurons [238]. This neuronal population is larger in females than males and plays a central role in the LH surge [239], [240]. Interestingly, a comparable increased sensitivity to a kisspeptin challenge in females was also observed in full body *Bmal1* knockout mice [166], where the mechanism for this increase remains unknown. Future work to determine

how neuronal network changes, with or without intact molecular clock function, impact GnRH neuron sensitivity to kisspeptin will be of interest. Although the kisspeptin challenge elicited a greater LH release in the $Vax1^{Vip}$ females than Ctrls, basal LH levels were comparable to Ctrls, and $Vax1^{Vip}$ females exhibited a decrease in circulating FSH. LH and FSH production and release are controlled in a great part by the pulsatile pattern of GnRH release [224], [241], [242]. Repeated blood sampling of $Vax1^{Vip}$ mice would have been an ideal approach to assess for changes in pulsatile hormone release, however, as $Vax1^{Vip}$ mice present with normal litter sizes, time to first litter, ovarian phase, which indicate overall normal ovarian function, and an increased activation of the stress axis, which is a suppressor of GnRH release [243], we decided against completing a LH and FSH pulse analysis due to the confounding effect of corticosterone on these data. In the future, we hope to better elucidate the relationship between stress and pulsatile hormones in this mouse model.

VAX1 in postnatal VIP neurons regulates female depressive-like behavior

Changes in the neuroendocrine regulation of FSH release from the pituitary in $Vax1^{Vip}$ mice is a potential pathway causing the reduction in estrogen of these mice. FSH is a limiting factor in the conversion of testosterone into estrogen in the granulosa cells of the ovary [244], [245], from where estrogen enters the general circulation. It is important to note that both FSH and estrogen are circadian [246], [247], thus a limit of this study, with blood sampling at a single time point, is our inability to assess if the reduction in these hormones might be due to a phase shift in hormone release. While primarily associated with its role in reproduction, estrogen has a multitude of functions, including altering the sensitivity of neuronal circuits [248], [249], regulating the activity of the stress axis [250], and displaying strong correlations with mood [251]–[253]. Low estrogen has been correlated with depressive-like behaviors in women [254]. In rodents, increasing estrogen has been shown to exert an anti-depressant-like effect during the Porsolt forced swim test, a test that is thought to reflect on depressive-like behavior in rodents [255], [256]. Additionally, an imbalance of progesterone and estrogen is associated with a higher incidence of mood disorders, including premenstrual dysphoric disorder (PMDD), a depressive disorder that presents with severe physical and physiological symptoms during the luteal phase of the menstrual cycle [257]–[260]. In addition to

recapitulating the low estradiol (or imbalance of progesterone and estrogen) as a risk factor for mood disorders, Vax1^{Vip} females also display another hallmark of PMDD, weakened SCN output [261], [262]. Excitingly, Vax1^{Vip} females (but not males, who have normal testosterone levels) have increased depressive-like behavior. Taken together, these data point to a novel role of VAX1 in regulating VIP neuron modulation of mood and the reproductive axis and raise the potential of Vax1^{Vip} females to serve as a new model for mood disorders that are tied to reproductive cycles, such as PMDD. Furthermore, VIP-and AVP-expressing neurons contribute to the regulation of the stress axis [263]–[266]. Stressful situations result in an increase in signal from the hypothalamus, which translates to higher levels of corticosterone via activation of the hypothalamic-pituitary-adrenal (stress) axis. Notably, the hypothalamus is comprised of several nuclei, including the SCN [267] and the PVN [268], [269], with varying roles and contributions to the stress axis. Within the SCN, reductions in *Vip* have been correlated with increased stress [270], whereas the VIP neuron target, the PVN, is a central relay station of the stress axis [205], [271]. Thus, the reduction of *Vip* in the SCN of Vax1^{Vip} females is a likely contributing factor in the increased corticosterone levels found in Vax1^{Vip} females, both at baseline and in response to a stressor. Specifically within the PVN, AVP is known to stimulate the stress axis [272], [273], and AVP expression in the PVN is comparable between control and VIP knockout males [274]. This suggests that the reduction in VIP in the SCN of Vax1^{Vip} mice may not be driving the changes in *Avp* mRNA within the PVN, but more likely is the result of other VAX1 targets that impact VIP neuron communication with PVN neurons, such as GABA [275], [276].

Conclusion and summary

Due to the abundance of VIP throughout the brain and body, it is difficult to study subsets of neurons expressing VIP without invasive surgery, which can lead to damaged brain tissue and a variety of other complications. In this study, we leveraged the close to 100% overlap of *Vax1* expression specifically within SCN *Vip* neurons, to generate a conditional knockout mouse model to study this subset of VIP neurons. We found that deletion of *Vax1* from SCN VIP neurons results in mice with altered circadian rhythms. Excitingly, Vax1^{Vip} females had disrupted reproductive axis function, low estrogen, high corticosterone, as well as in increase in depressive-like behaviors. Together, these data provide us with an

exciting new model to study the genetic and neurological overlap between circadian disruption, female reproductive health, and depressive-like behaviors.

CHAPTER 4: DICHOTOMIC EFFECT OF ROTATING LIGHT SHIFTS ON FEMALE NEUROENDOCRINE CONTROL OF THE REPRODUCTIVE AXIS IN MICE

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Author Contributions: All authors provided contributions to study conception and design, acquisition of data or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published. Here are the most important contributions of each author: H.M.H., A.M.Y., and B.M.V.L. designed the study. Data were collected by B.M.V.L., A.M.Y., A.K.M.S., K.Y.J., K.J., and D.N. Analysis was carried out by A.M.Y., K.J., B.M.V.L., and H.M.H. The manuscript was written by B.M.V.L., A.M.Y., and H.M.H. All authors approve and take final responsibility for this article.

Abstract

Shift work disrupts circadian rhythms and is associated with a range of health issues, including menstrual irregularities, infertility, and pregnancy complications in women. However, not all women exposed to shift work experience these reproductive disruptions, and the underlying physiological differences remain unclear. To explore this variability, we exposed female PER2::LUC circadian reporter mice to a rotating light (RL) paradigm designed to mimic human shift work. In contrast to stable light:dark (LD) controls, half of the RL-exposed mice developed irregular estrous cycles (RL-A), while the other half retained regular cycling (RL-C). Compared to LD mice, both RL-C and RL-A mice displayed significant mistiming of the time of peak PER2::LUC expression in the reproductive axis, including the ovary and uterus. Functional assays revealed that RL-A mice as compared to LD and RL-C mice had a blunted luteinizing hormone release in response to gonadotropin-releasing hormone administration, reduced follicular development, and decreased progesterone levels, indicating compromised neuroendocrine regulation of reproduction. Despite elevated corticosterone, RL-A mice did not exhibit increased depressive-like behaviors compared to LD and RL-C mice. Surprisingly, both RL-A and RL-C mice had

comparable fertility to LD mice, with a significant reduction in litter sizes. This work provides mechanistic insight into how shift work may differentially impact female reproductive health and highlights the need for individualized approaches to managing reproductive risks in women working nontraditional schedules.

Introduction

Over 26 million Americans participate in shift work (working outside the working hours of 9 am to 5 pm) annually. Shift work negatively impacts many aspects of health including increasing the risk for cancer [277]–[279], heart disease [280]–[282], and female fertility [60], [232], [283]. While many studies have focused on the impact of shift work on female fertility [6], [283], [284], shift work also negatively impacts daily life through changes to the menstrual cycle [285], [286], where shift workers have increased risk for both shortened and lengthened menstrual cycles, irregular menstrual cycles, and increased dysmenorrhea (discomfort in the menstrual cycle) [287]–[290]. However, not all female shift workers develop menstrual cycle issues [287], [291], though the physiological differences between those that develop problems and those who do not have not yet been identified.

Female reproductive success is regulated by the hypothalamic-pituitary-gonadal (HPG) axis [115]. Within the brain's hypothalamus, gonadotropin-releasing hormone (GnRH) neurons project to the pituitary gland, prompting the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) [292]. LH and FSH promote follicular development and maturation and prompt ovulation via a characteristic surge of LH [187], [293]. The HPG axis receives input from numerous brain regions, including the brains circadian pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is instrumental in the process of coordinating internal timekeeping and 24 hour processes (circadian rhythms) to external cues, like the day/night cycle [22]. To do this, the SCN receives signals from the retina [71] that project to vasoactive intestinal peptide (VIP) neurons in the SCN. VIP neurons in turn project to GnRH neurons, which are crucial for HPG axis function. This coordination of timed hormone release to impact target tissue function requires a precise synchrony among each tissue within the HPG axis. Disruption to HPG axis synchrony (or alignment) leads to reduced reproductive function, including menstrual disorders and problems with ovulation [115]. Neuronal signals from the improper timing of these cues, such as

frequent exposure to light at night through shift work or staying up late with lights on, disrupts the reproductive axis by altering the timing of the neurological signals that then cascade down the remainer of the HPG axis [294]. Though the exact effects of light-induced circadian misalignment on individual tissues of the reproductive axis have yet to be explored, much research has focused on the deregulated hormones that result from disrupted circadian rhythms, increasing the risk of hormone-related breast cancer in women [295], [296].

Shift work can disrupt the HPG axis through mistimed light exposure. Rodent studies have found that shift work-like light cycles increase the prevalence of irregular estrous cycles in mice. Nine month exposure to lights that rotate between 10 hour advances and 10 hour delays every 3-4 days results in about 50% of the female mice exhibiting irregular estrous cycles [56]. Another group found that another light paradigm with a 12 h shift light shift every 3, 6, or 12 days for 36 days also results in irregular estrous cycles that persist for up to three weeks after the lights are restored to conventional 12 hours light, 12 hours dark [297]. Interestingly, similar to human reports, where only a subset of women develop menstrual disorders following shiftwork, not all of the mice in these studies developed irregular estrous cycles. However, no work has yet been done to determine the underlying physiological differences between the mice that develop irregular cycles and those that maintain estrous cyclicity when exposed to rotating lights.

The goal of our study was to identify a physiological marker that could differentiate which female mice would develop irregular estrous cycles in response to rotating lights and which would maintain normal estrous cyclicity. Identifying potential biomarkers that could allow us to predict which mice may develop irregular estrous cycles and potentially treat the root cause of these issues.

Methods

Mice

All methods described here have been approved by the Institutional Animal Care and Use Committee of Michigan State University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Period2::Luciferase (PER2::LUC) mice were purchased from JAX (strain B6.129S6-Per2tm1Jt/J, #006852, https://www.jax.org/strain/006852). Mice were single housed with a

running wheel under a 12 h light–dark cycle (LD), with food and water ad libitum. Mice were euthanized by cervical dislocation. Experimental mice were 6–14 weeks of age at the start of experiments.

Experimental Light Conditions and Timeline

The LD lighting paradigm began with a 14-day baseline of stable Light:Dark (LD)12:12 light exposure with lights on at 0400h (ZT0) and lights off at 1600h (ZT12). Control mice were maintained on the stable LD12:12 for the duration of experiments. For the shiftwork lighting schedule, mice underwent several shiftwork cycles, which consisted of a combined phase delay and advance (8 days) where the first four days were "delayed" by a 6 h shift from the time of lights on followed by four days of an "advanced" cycle, with a 6 h shift such that lights returned to baseline time for 40-60 days. This paradigm is referred to as rotating lights (RL).

Estrous cycling

Estrous cyclicity was monitored by vaginal lavage with 20 µl H_2O daily between ZT3 and ZT5 for 16-18 days [143], [298], [299]. The lavage solution was dried on a slide and stained with 0.5% methylene blue. Cytology was visually examined and scored. Following the 14 days of adjustment period, baseline estrous cycling was assessed (14 days) before beginning the shiftwork lighting scheme. Following the baseline estrous cycling, females were exposed to 6 shiftwork cycles and then another 14 days of estrous cycling. Mice were determined to have normal estrous cycles if they progress through estrus, metestrus, diestrus, and proestrus in order during the 14-day span and were determined to have abnormal cycles if they failed to progress through estrus, metestrus, diestrus, and proestrus in order during the 14-day span measured.

Pregnancy Experimental Timeline

For pregnancy experiments, single-housed female mice were exposed to the baseline LD lighting paradigm followed by 8-9 weeks of rotating lights (RL). Following this exposure, an experienced male was introduced to the cage for two weeks. Females were examined for copulatory plugs each morning and the presence of a plug was considered gestational day (GD) 0. After two weeks, the male was removed from the cage. Samples were then collected either at ZT3 on GD14 as determined by the day of plug and

confirmed by Theiler staging the pups or during active labor as determined by at least one pup having been born but pups remaining in the uterus.

Wheel-Running Behavior

During shiftwork lighting, wheel running behavior was collected and analyzed as previously reported [131], [143]. Female mice were single-housed in light and temperature controlled circadian cabinets (standard mouse circadian cabinet, Actimetrics, Wilmette, IL) within polypropylene cages (33.2 × 15 × 13 cm) containing a metal running wheel (11 cm diameter). All mice were acclimated to running wheels for 2–5 days prior to experimental start. Mouse locomotor activity rhythms were monitored with a ClockLab data collection system (Version 3.603, Actimetrics, Wilmette, IL) through the number of electrical closures triggered by wheel rotations. Light intensity varied between 268 and 369 lux inside the mouse cage with wheel. Cage changes were scheduled at 2-week intervals. Wheel-running activity was analyzed using ClockLab Analysis 5 (Version 6.0.54, Actimetrics Software) and complied into 6-min bins by persons blind to experimental group. Daily onset of activity was defined as the first time when activity was counted for at least 1 h after at least 4 h of inactivity, and activity offset was defined as 1 h of inactivity following 4 h of activity.

Sucrose Preference Test

Following either the RL or LD lighting protocol, female mice received two water bottles, one with regular drinking water and one with 1% sucrose water for 7 days as described by [300]. The bottles were weighed daily and switched places daily to reduce any effects due to the location of the bottle. The value reported is the percent of sucrose water consumed out of the total weight of water consumed.

Three-Chamber Social Preference Test

Following either the RL or LD lighting protocol, during days 3 and 4 of phase delays, female mice in metestrus at ZT 12 (lights off) were transferred to a behavioral room and allowed to habituate under red lights for 1 hour prior to the Three-Chamber Social Preference Test [301]–[303]. Following the habituation, mice were placed in a three-chamber behavioral apparatus measuring 102 cm (L) × 47 cm (W) × 45 cm (H) with the walls between chambers made of transparent plexiglass and each internal wall containing a small

opening. Each of the two end chambers contained a 10.2 cm (diameter) × 10.8 cm (height) transparent plastic cup with 1 cm gaps between bars. For the habituation phase, these cups remained empty. During the habituation phase, mice explored the apparatus for 10 minutes and were then returned to their home cage for a 5 minute rest period. During the pre-test phase the two plastic cups contained crumbled paper balls of comparable size and during the test phase, the balls were replaced with either a non-social stimulus (a simple lego structure) or a sex and age matched non-littermate mouse as previously described [301]. All test phases lasted 10 minutes with 5 minute rest periods between test phases. Videos were scored by an observer blinded to the experimental group. The time spent in each chamber (with the non-social stimulus, center, and with the social stimulus) was recorded. The social preference index was calculated from the following formula: [(time in the social stimulus chamber – time in the non-social stimulus chamber)/(time in the social stimulus chamber + time in the non-social stimulus chamber)].

Porsolt Forced Swim Test

Porsolt Forced Swim tests were conducted and analyzed as described [143], [197]. Following either the RL or LD lighting protocol, metestrus female mice were placed in a transparent 7 by 24-inch cylinder filled 2/3 full with 20-21°C water. Swim tests were completed during the mice active phase and started at ZT13. Mice were recorded in dim red light for 6 minutes. Videos were scored by an observer blinded to the experimental group using the sampling method. The mouse was determined to be either floating or swimming every 30 seconds for 5 minutes, with the first minute of each video going unscored and serving as an adjustment period. The percent of intervals where the mouse was observed to be floating is reported.

Tissue Explant PER2::LUC Bioluminescence Recordings

To measure tissue level circadian rhythms, we analyzed explants from PER2::LUC reporter mice as previously described [143], [152]. Mice were euthanized at ZT3 3-4 days following both phase advances and phase delays. Following euthanasia, the pituitary, brain, uterus and ovary were removed from all mice and placed in a semi-frozen 1× Hank's buffered salt solution (HBSS, 14065-056, Gibco). Using a dissection scope the ovaries were isolated and the uterus dissected into pieces of ≈ 4 mm^2 (~2 × 2 mm). To prepare

the uterine pieces, the whole uterus was cleaned removing fat and placenta attachment tissue and cut in the longitudinal direction of the uterine horn. Using a ruler, 2 × 2 mm uterine strips were collected midway between the cervix and the ovary near the placental attachment and placed with the endometrium side down onto the MiliCell membrane (MilliCell, PICM0RG50; MilliporeSigma, Burlington, MA). Ice-cold brains were sliced coronally on a vibratome (Leica VT 1200S) at 300 μm. After sectioning on the vibratome, a dissection scope was used to identify the appropriate brain regions. The SCN was located through anatomical identification (Franklin & Paxinos, 2008). The SCN was dissected as previously described [304], [305]. MilliCell membranes were placed in 35-mm dishes (Nunc, Thermo Fisher Scientific, Rochester, NY) containing 1.5 ml of 35.5°C recording medium (Neurobasal, 1964475, Gibco) supplemented with 20 mM HEPES (pH 7.2), B27 supplement (2%; 12349-015, Gibco), 1 mM luciferin (luciferin sodium salt; 1-360242-200, Regis, Grove, IL), and antibiotics (8 U/ml penicillin, 0.2 mg/ml streptomycin, 4 mM l-glutamine; Sigma-Aldrich). Dishes were sealed using vacuum grease and placed into a LumiCycle (Actimetrics, Wilmette, IL) inside a light-tight 35.5°C, 5% CO_2, non-humidified environmental chamber. The bioluminescence signal was counted every 10 min for 1.11 min for 6 days (days 1–6 of recording time). Data were normalized by subtraction of the 24 h running average from the raw data and then smoothed with a 1 h running average (Luminometer Analysis, Actimetrics) and analyzed blind to experimental group. During the initial ~24 h (day 0) in the LumiCycle, the PER2::LUC signal tends to decrease significantly prior to achieving a stable waveform [152]. In our analysis of PER2::LUC period, we exclude the first 24 h of recording to account for this. Incomplete data sets, as caused by loss of data points, other technical problems, or explants failing to show two PER2::LUC peaks (deemed arrhythmic as per [306]) were not included in the analyses. PER2::LUC phase was determined as the time of day of first PER2::LUC peak. PER2::LUC period was analyzed by the Luminometer Analysis software (Actimetrics) as the time difference in hours between the two peaks, with LM fit (damped sin) as the mathematical model. To determine the phase of the tissue, we utilized the time of peak signal on day 1 of recording (time of first peak). All phase data were converted to radian and are reported in degrees and reference to zeitgeber time (ZT) with time of lights on at ZT0 from the day of euthanasia.

Hormonal Challenges and Blood Hormones

Hormonal challenges were done using GnRH intraperitoneal (i.p.) injections at ZT3-4 in diestrus during day 3 or 4 of phase advances [143]. GnRH (Millipore Sigma, catalog #L7134, 1 μg/kg dose) diluted in sterile physiological saline (Intermountain Life Sciences, catalog #Z1376) was injected to diestrus females and blood was collected from the mouse from the tail vein before (time 0) and after i.p. injection at time points 5, 10, 15, 30, and 45 minutes. For all other serum hormone analyses, mice were killed by cervical dislocation and blood collected from the abdominal aorta between ZT3 and ZT6. Blood was allowed to clot for 1 hour at room temperature, then centrifuged and supernatant recovered (room temperature, 15 minutes, 2,600× g).

Hormone assays

Serum was stored at 80°C before analysis for progesterone at the Center for Research in Reproduction, Ligand Assay, and Analysis Core, University of Virginia (Charlottesville), by Luminex analysis for LH on MILLIPLEX MAP Mouse Pituitary Magnetic Bead Panel (Millipore Sigma #MPTMAG-49k) or a competitive enzyme-linked immunosorbent assay (ELISA) kit (EIACORT, ThermoFisher) for corticosterone. Coefficients of variance (CVs) were based on the variance of samples in the standard curve run in duplicate. Reportable range: Progesterone: 0.15–20 ng/ml, CV = <20%; LH: lower detection limit: 5.6 pg/ml, CV < 15%; corticosterone: lower detection limit: 0.87 μg/dl, CV = <20%. Samples were run in singlets.

Immunohistochemistry for VIP and GnRH

Tissues were collected between ZT3 and ZT5 3-4 days following phase advances from adult diestrus female mice on LD or rotating light cycle and fixed overnight at 4˚C in 60% ethanol, 10% formaldehyde, and 10% glacial acetic acid. Tissues were washed in 70% ethanol and embedded in paraffin. Single immunohistochemistry on 10 μm coronal brain sections embedded in paraffin was performed as previously described [126]. The primary antibody was rabbit anti-VIP (Immunostar #20077, 1:1000, RRID:AB_572270) or anti-GnRH (ThermoFisher Scientific#PA1-121, 1:500, RRID:AB_325077). Sections were incubated in 1:300 secondary anti-rabbit IgG (Vector Laboratories, #BA-1000). Secondary

antibodies were purchased from Vector labs, and colorimetric VIP (purple staining) and DAB (brown staining) assays (Vector laboratories) revealed the primary antibodies. VIP staining was quantified automatically in six sections evenly distributed between Bregma -0.22 and -0.58 by the Lionheart (BioTek Lionheart FX Automated Microscope) Gen5 software (version 3.10 BioTek) as previously described [121]. GnRH staining was quantified by measuring staining intensity in a 200 μm by 500 μm area encompassing the median eminence at Bregma -2.06 by the Lionheart (BioTek Lionheart FX Automated Microscope) Gen5 software (version 3.10 BioTek).

Statistical Analysis

Data were analyzed using GraphPad Prism 10 (Graph Pad Software, La Jolla, CA) or RStudio (Version 2024.12.1+563 (2024.12.1+563)). Significant differences were designated as $p < 0.05$. Wheel running activity (phase angle of entrainment and period) was analyzed via a repeated measures mixed effects model (REML) followed by Bonferroni post hoc analyses where appropriate. Estrous cycle lengths, PER2::LUC period, hormone data, follicle counts, and behavioral tests were analyzed via one-way ANOVA. Post-hoc tests were completed using Tukey's multiple comparison test. PER2::LUC timing of first peak phase relationships were analyzed via a Rayleigh test of Uniformity, followed by pairwise comparisons, Watson's Two Sample Test of Homogeneity using Bonferroni's correction to accommodate familywise error rate, where appropriate. Phase data were reported in degrees and standard error (SE). For comparing percentages, a Z score calculator was used. All data passed normality testing. Statistical analyses for outliers (Grubbs' test) were conducted on all data sets, and no outliers were identified.

Results

Rotating lights result in abnormal estrous cycles in 50% of female mice

To determine if our shiftwork light-paradigm, termed rotating light (RL), impacted estrous cycles, we single-housed female mice with running wheels and exposed them to either LD lights or the rotating light paradigm (RL) for 40 days (Fig.4.1A). Mice were determined to have normal estrous cycles if they progress through estrus, metestrus, diestrus, and proestrus in order during the 14-day span and were determined to have abnormal cycles if they did not (Fig. 4.1B,C). Mice exposed to LD lights maintained

normal estrous cycles. However, after 40 days of exposure to rotating lights, 50.0% of female mice experienced abnormal estrous cycles, defined as the failure to progress through estrus, metestrus, diestrus, and proestrus in order during the 14-day span measured (Fig. 4.1D,E). Given this, we categorized the RL mice into two subgroups: RL-cyclic (RL-C), which were mice who displayed estrous cycles comparable to LD and mice with RL-abnormal (RL-A), which were mice who experienced abnormal estrous cycles. There was no difference in cycle length between LD mice and RL-C mice; however RL-A mice were determined to have significantly longer estrous cycles than both LD and RL-C mice (One-way ANOVA, $p < 0.0001$; Fig. 4.1F).

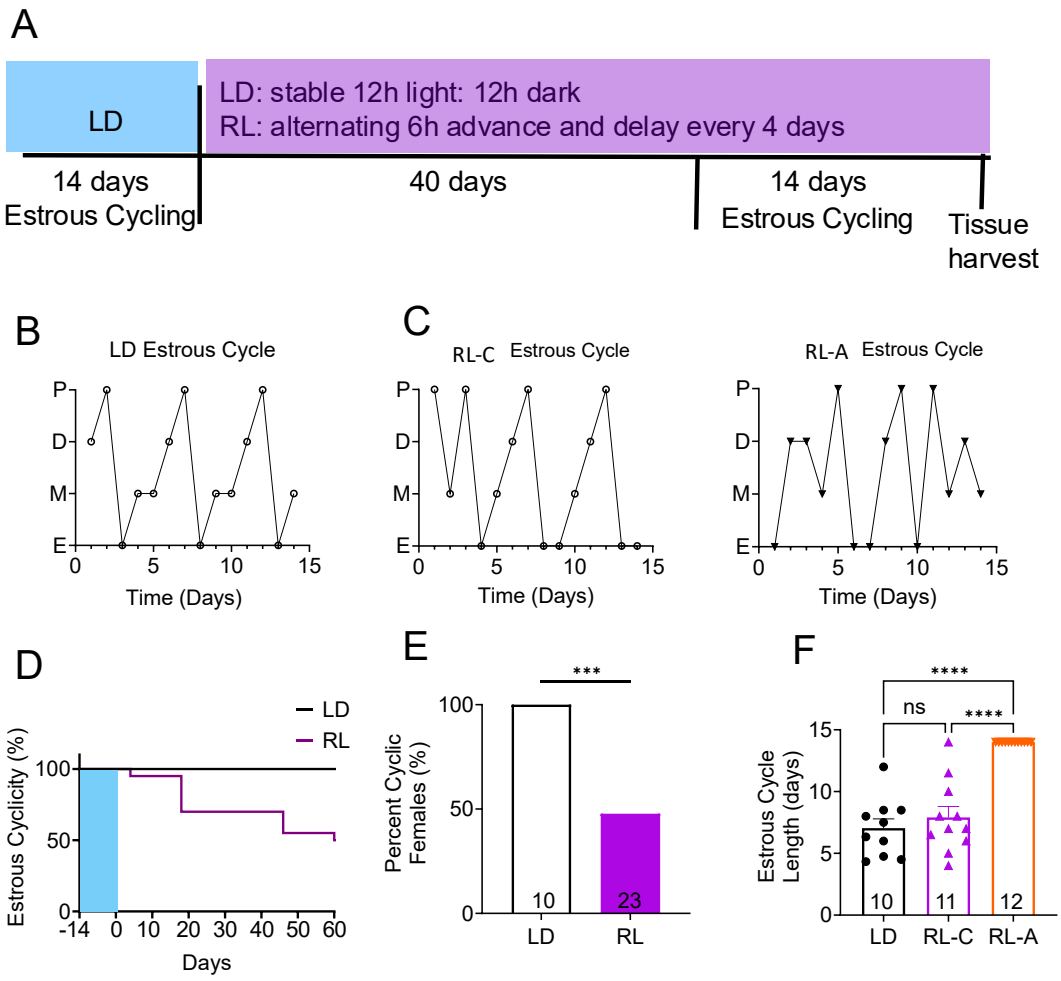

Figure 4.1. Rotating lights paradigm results in irregular cycles in around 50% of female mice. A) Timeline for the experiments conducted where blue is representative of stable light:dark (LD) lighting conditions and purple is representative of experimental mice exposure to rotating lights (RL) while controls remain in LD. B) Representative example of a normal estrous cycle in a mouse on LD. C) Representative example of a normal and abnormal estrous cycle on RL. D) Survival curve showing the percentage of mice maintaining estrous cyclicity following the baseline LD period (blue) period, where day 0 begins exposure to LD (control mice) or RL (experimental mice). E) The percentage of females exhibiting normal estrous cycles when exposed to either LD or RL. Z-score, ***, $p < 0.001$, $n = 10$-20. G) Comparison between the average cycle length of LD mice, RL-cyclic (RL-C) and RL-abnormal (RL-A) mice. One-way ANOVA, ****, $p < 0.0001$.

Female mice exposed to RL fail to adapt to phase advances

As sex hormones can impact female entrainment to a light shift [307], we wanted to determine if the differences in estrous cycles would differently impact the entrainment of RL-A and RL-C mice to the rotating lights. We examined the actograms of LD (Fig. 4.2A), RL-C (Fig. 4.2B), and RL-A (Fig. 4.2C) during both stable LD, and after light advances and delays. We found that LD mice remained entrained to the stable LD lighting paradigm, but both RL-A and RL-C mice showed a significant difference from LD mice in the phase angle of entrainment during phase advances, but not phase delays RL-A (Mixed-effects analysis, $p < 0.05$; Fig. 4.2D), suggesting that, independent of cyclicity, mice do not adapt behaviorally to phase advances, but do adapt to phase delays.

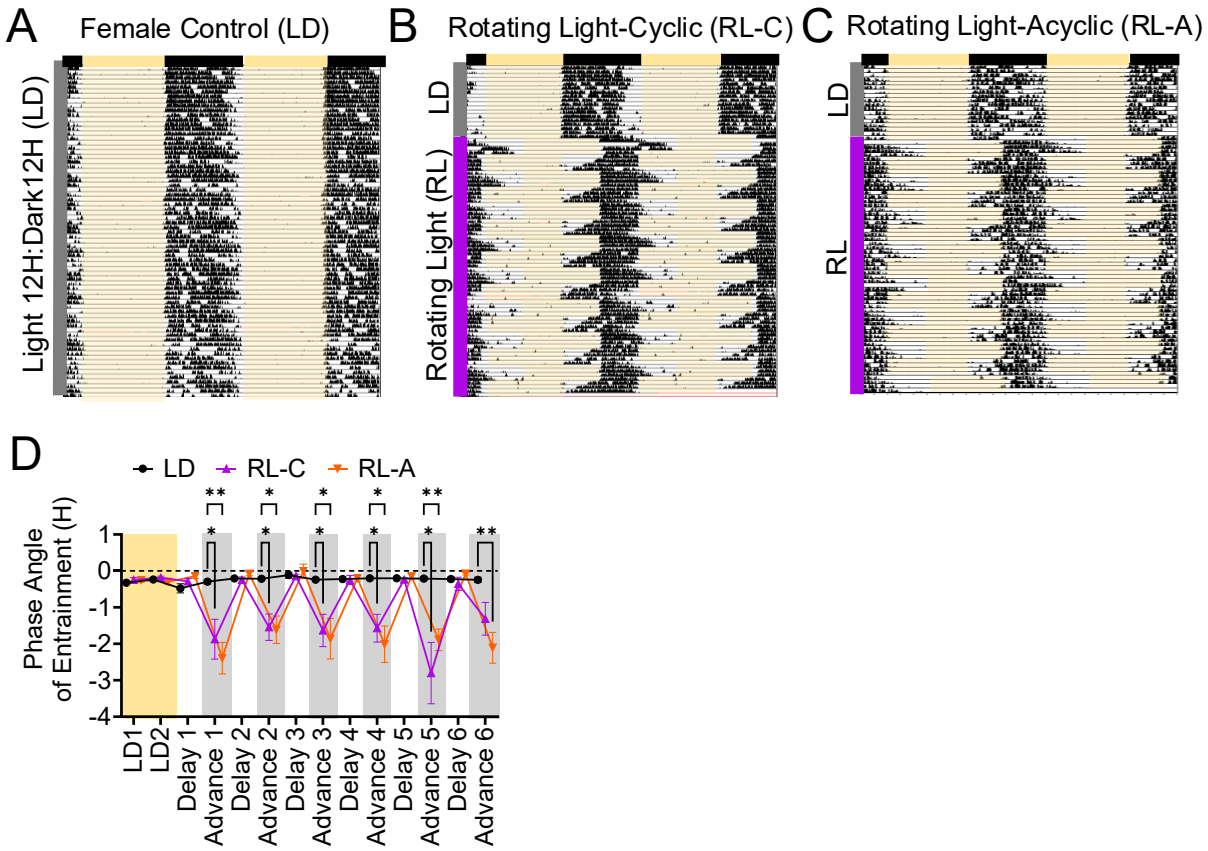

Figure 4.2. Both RL-A and RL-C mice fail to entrain to phase advances but entrain to phase delays. Representative, double-plotted actograms for A) LD females, B) RL-C females and C) RL-A females. D) phase angle of entrainment for LD (black), RL-C (purple), and RL-A (orange) mice. Mixed-effects Analysis, n = 9-10. *, $p < 0.05$; **, $p < 0.01$.

RL mice exhibit disruption in HPG axis tissue circadian rhythms.

While wheel running data demonstrates behavioral adaptation, SCN entrainment may be masked by light during phase delays, which may result in an appearance of behavioral entrainment but without true entrainment occurring [202], [308]. To determine if HPG axis tissues are entrained to the RL paradigm, we used the PER2::Luciferase circadian reporter mouse model [46] to record tissues-level Per2 rhythms in LD, RL-C, and RL-A mice. In the SCN, we found no differences in our explant success (Z-score, $p > 0.05$; Fig. 4.3A) or the period of the SCN (One-way ANOVA, $p > 0.05$; Fig. 4.3B). The phase of the SCN, defined as the time of day of the first peak, passed the Rayleigh's test of Uniformity, indicating a clustering (aligned time of day/phase) for LD SCN and RL-C SCN. The RL-A did not pass the Rayleigh's test of Uniformity, indicating a lack of clustering of the time of first peak (Fig. 4.3C). There was a significant difference in phase between the LD SCN and the RL-C (advance) SCN (Watson's Two Test, $p < 0.05$. In the pituitary gland, we found a significant reduction in explant success in the RL-A (advance) females (Z-score, $p = 0.01$; Fig. 4.3D). However, there was no difference in the period (One-way ANOVA, $p > 0.05$; Fig. 4.3E). Neither the RL-C (advance) nor RL-A (advance) mice passed the Rayleigh's test (Fig. 4.3F). There was a significant difference between the phase of the LD pituitary gland and the RL-C (delay) mice (Watson's Two Test, $p < 0.05$). At the level of the ovary, we did not find a significant difference in the explant success rate (Z-score, $p > 0.05$; Fig. 4.3G) or the period (One-way ANOVA, $p > 0.05$; Fig. 4.3H). Only the RL-A (delay) ovary did not pass the Rayleigh's test, and there was a significant difference in phase between the LD ovaries compared to the RL-C (advance) and RL-C (delay), as well as between the LD ovary and the RL-A (advance) (Watson's Two Test, $p < 0.05$; Fig. 4.3I). Similarly, the uterus showed no differences in the success rate (Z-score, $p > 0.05$; Fig. 4.3J) or the period (One-way ANOVA, $p > 0.05$; Fig. 4.3K), and all but the RL-C (advance) uterus passed the Rayleigh's test (Fig. 4.3L). There were significant phase differences between RL-A (delay) uteri versus LD, RL-C (delay), and RL-A (advance). RL-C (delay) mice differed from RL-A (delay) mice (Watson's Two Test, $p < 0.05$), indicating mistiming in the uterus of both RL-A and RL-C mice.

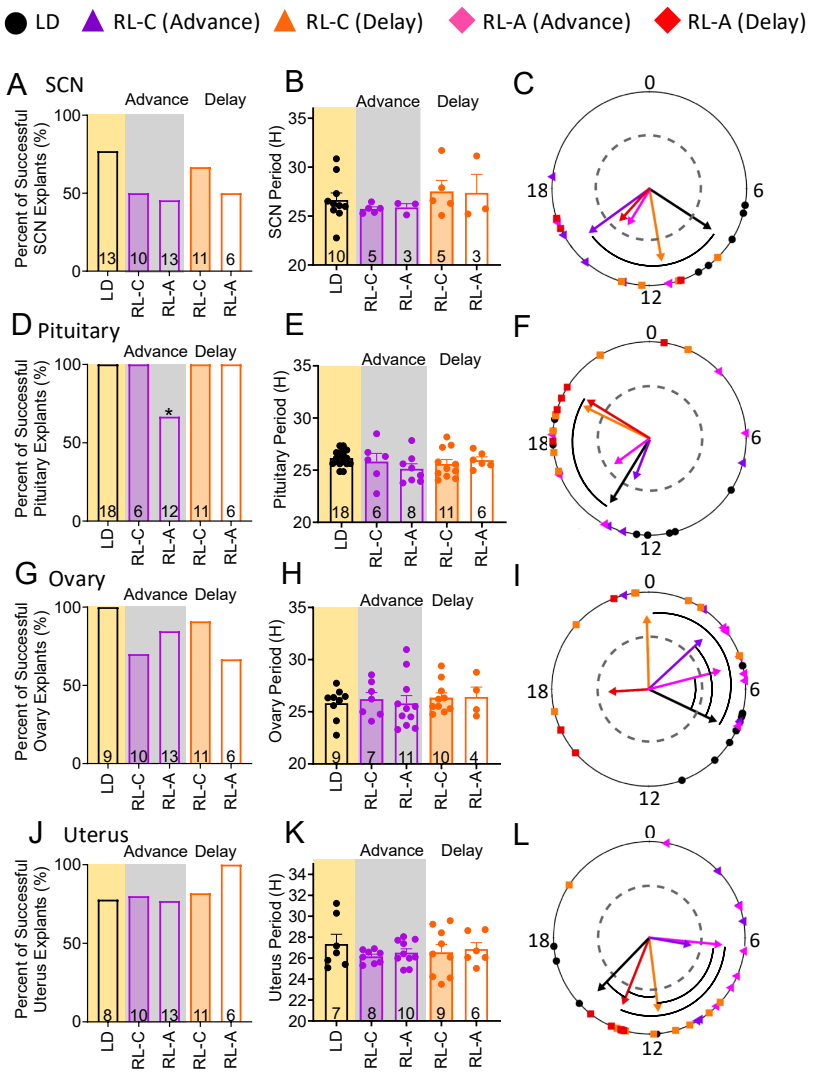

Figure 4.3. RL mice exhibit significant PER2::LUC desynchrony in the tissues of the HPG axis. A, D, G, J) Explant success of indicated tissues. Explant success was calculated using a Z-score. *, $p < 0.05$. B, E, H, K) Explant period. Two-way ANOVA, $p>0.05$. C, F, I, L) Circular graph of the phase of the first peak of successful explants from LD, RL-A, and RL-C mice. Yellow shading indicates samples were collected at ZT3 on LD; grey shading indicates tissues were collected at ZT3 3-4 days after an advance, and white that the collection was at ZT3 3-4 days after a delay. An arrow crossing the dotted line indicates significance in the Rayleigh's test. Black lines indicate significant differences between groups.

RL-A mice have reduced functionality of the HPG axis at the level of the pituitary compared to LD and RL-C mice

Due to the mistiming of phase observed in the ovary and uterus in both RL-C and RL-A mice compared to controls, we hypothesized that the physiological differences giving rise to the irregular estrous cycles in the RL-A mice may be due to differences in peptide expression in the SCN. As VIP plays a role in both circadian adaption to mistimed light exposure [74] and regulates the HPG axis [14], we asked if RL would impact VIP expression within the SCN. After 40 days of either LD or RL, we conducted estrous cycling to determine which mice were RL-C and which were RL-A, followed by a GnRH challenge and tissue collection (Fig. 4.4A). Using immunohistochemistry, we examined the expression of VIP in the SCN of LD, RL-C, and RL-A female mice (Fig. 4.4B). We used a semi-quantitative method to determine if there were detectable differences between the tissues by measuring the staining intensity (Fig. 4.4C). Unfortunately, multiple samples were lost during the staining process due to a faulty batch of slides, resulting in a low sample size. A cohort of controls is currently underway to generate new samples. VIP can promote activation of GnRH neurons, which in turn regulates the release of LH [14]. To determine if RL impacted GnRH levels, we completed immunohistochemistry for GnRH at the median eminence, the primary projection site of GnRH terminals, in LD, RL-C, and RL-A mice (Fig. 4.4D) and used semi-quantitative methods to determine any obvious differences (Fig. 4.4E). Again, multiple samples were lost, and conclusions could not be made from these data.

Independent of VIP or GnRH levels, the pulsatile release of GnRH defines pituitary function [309], [310]. To determine if RL impacted the LH release from gonadotropes, we injected GnRH and measured the resulting LH levels in the blood of the mouse at ZT3 in diestrus. In response to the GnRH injection, LH significantly increased in both the LD and RL-C mice with no significant differences between the two. However, the RL-A mice exhibited a reduced LH release profile (Fig. 4.4F). Overall, RL-C had a significant reduction in the total LH released in response to the GnRH challenge (One-way ANOVA, $p = 0.0079$; Fig. 4.4G).

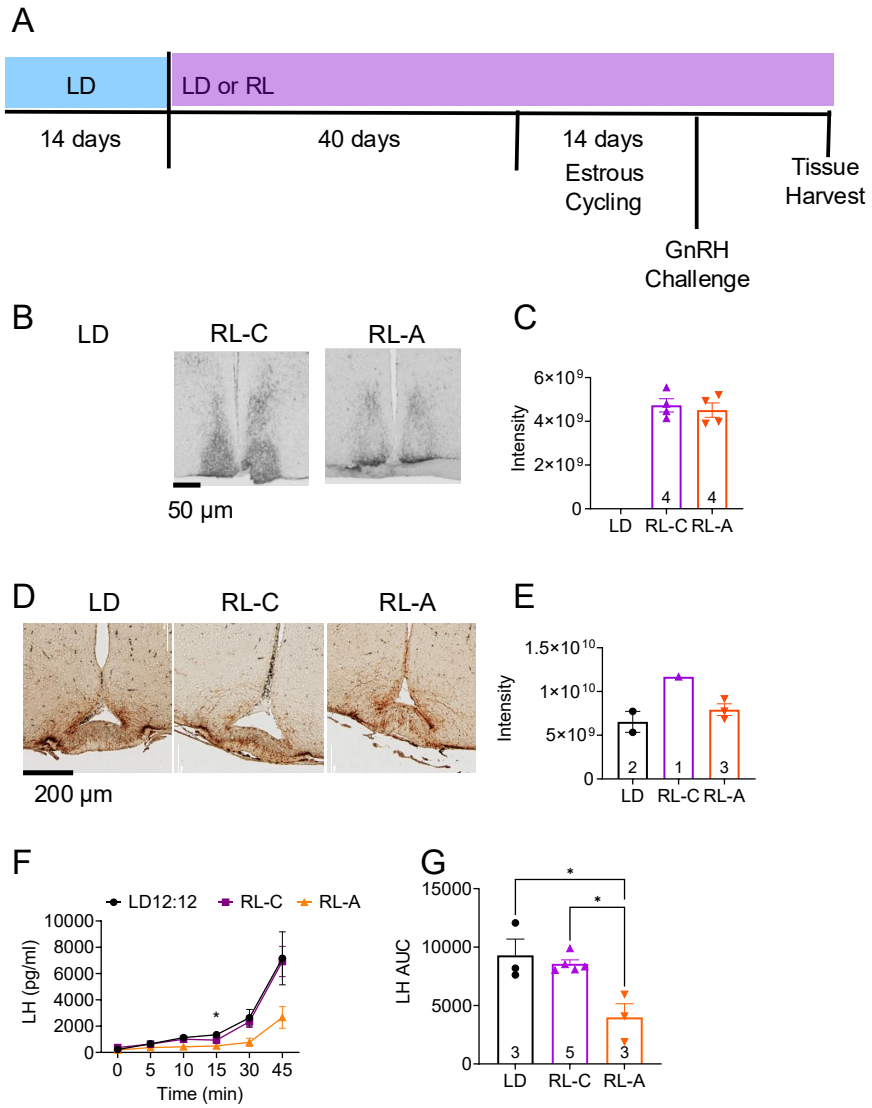

Figure 4.4. RL-A mice have a reduced LH release to a GnRH challenges. A) Timeline for the experiment where blue indicates stable LD lighting for both groups while purple indicates RL for experimental mice and stable LD for controls. B) immunohistochemistry for VIP in the SCN of RL-C and RL-A mice. LD mice data are missing due to technical issues. Scale bar = 50 um C) Intensity quantification of RL-C and RL-A female mice. D) Immunohistochemistry for GnRH in the median eminence of the brain in LD, RL-C, and RL-A female mice. Scale bar = 200 um E) Intensity quantification of GnRH in the median eminence of LD, RL-C, and RL-A mice. F) Time course of LH following a GnRH challenge. Mixed-effects analysis, n = 3-5. * $p < 0.05$ G) Total LH released (AUC) in 45 minutes in response to a GnRH challenge. One-way ANOVA. * $p < 0.05$.

Follicle counts and progesterone release are reduced in RL-A mice compared to LD and RL-C mice

LH maintains ovarian function, including the regulation of sex-steroid hormone release and follicular development [311]. To determine if the changes in pituitary function impacted the ovary, we counted the number of each follicle type present in LD, RL-C, and RL-A ovaries (Fig. 4.5A-C). When examining the percentage of each follicle type out of the total number of follicles, we found that there was a significant decrease in primary follicles in both RL-C and RL-A mice compared to LD mice (One-way ANOVA, $p = 0.0032$; Fig. 4.5D). In addition, we found a significant decrease secondary follicles (One-way ANOVA, $p = 0.0301$; Fig. 4.5E), and pre-antral follicles in RL-A mice compared to LD mice (One-way ANOVA, $p = 0.0383$; Fig. 4.5F), as well as an increase in atretic follicles in RL-A mice compared to LD mice (One-way ANOVA, $p = 0.0157$; Fig. 4.5G). There were no differences between LD, RL-C, or RL-A mice in corpus luteum counts (One-way ANOVA, $p > 0.05$; Fig. 4.5H). To determine if either the changes in follicles or the alterations in pituitary sensitivity to GnRH affects sex-steroid hormone release from the ovary, we quantified progesterone present in the serum at ZT3 during diestrus. We found comparable progesterone levels between the LD mice and the RL-C mice; however, the RL-A mice had a significant reduction in circulating progesterone compared to the LD mice (One-way ANOVA, $p = 0.0203$; Fig. 4.5I), indicating reduced corpus luteum function in the ovary.

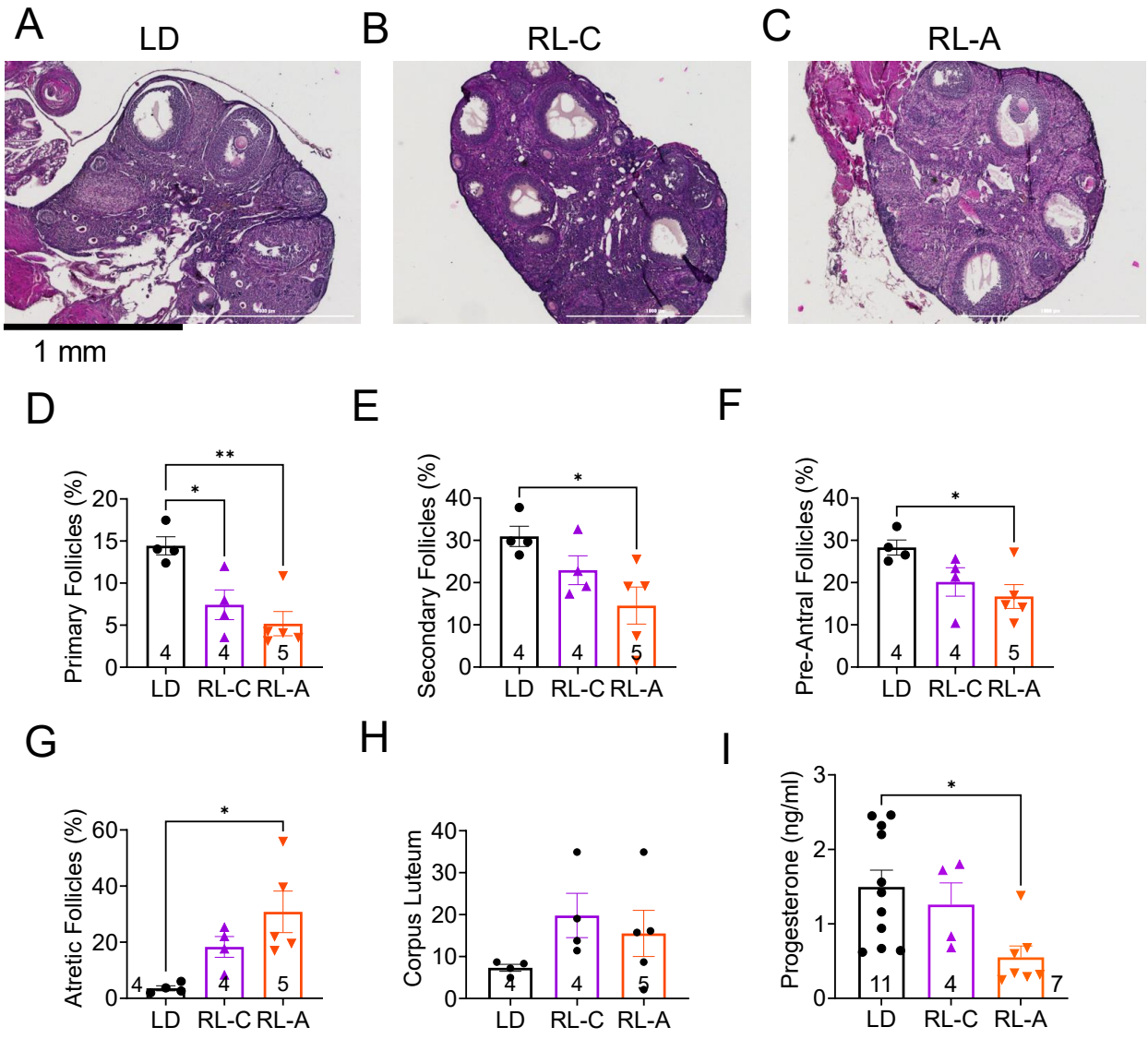

Figure 4.5. RL-A mice have an increase in atretic follicles compared to LD and RL-C mice. A, B, C) Representative Hematoxylin and Eosin stained ovaries from LD, RL-C and RL-A female mice. Scale bar = 1 mm. D) Percent primary follicles/ovary in LD, RL-C, and RL-A mice, D) secondary follicles, F) pre-antral follicles, G) atretic follicles, and H) corpus luteum. One-way ANOVA. * $p < 0.05$; ** $p < 0.01$. I) Serum progesterone from ZT3 during diestrus in LD, RL-C, and RL-A female mice 3-4 days following a phase advance. One-way ANOVA. *, $p < 0.05$.

Rotating lights do not increase depressive-like behaviors

Low levels of progesterone in mice can increase depressive-like behaviors and supplementation of progesterone can rescue these behaviors [191], [258], [260]. In animal models of high stress, low levels of progesterone have been correlated with higher levels of corticosterone, a hormone known for its involvement in stress [312], [313]. To determine if the RL-A mice, which have lower levels of progesterone, also exhibit increases in stress and depressive-like behaviors, we measured corticosterone and used a series of behavioral tests to assess hedonic reward (sucrose preference test), behavioral despair (forced swim test), and social reward (sociability; three-chamber social preference test). Following the LD or RL paradigm, we conducted estrous cycling to determine which mice are RL-C and which are RL-A, followed by the described behavioral tests (Fig. 4.6A). We found comparable levels of corticosterone between LD and RL-C mice, but RL-A mice had a significant increase in serum levels of corticosterone compared to both LD and RL-C mice (One-way ANOVA, $p = 0.0006$; Fig. 4.6B). The sucrose preference test, used to measure anhedonia [300], resulted in no significant differences between the LD, RL-C, and RL-A mice (One-way ANOVA, $p > 0.05$; Fig. 4.6C). Similarly, no differences were observed in the forced swim test, a behavioral test used to measure behavioral despair (One-way ANOVA, $p > 0.05$; Fig. 4.6D). Finally, we used the three-chamber social preference test to measure sociability, which is often reduced in depressive disorders. Our results revealed a significant decrease in social interaction in the RL-C mice as compared to the LD and RL-A mice (One-way ANOVA, $p = 0.0264$; Fig. 4.6E).

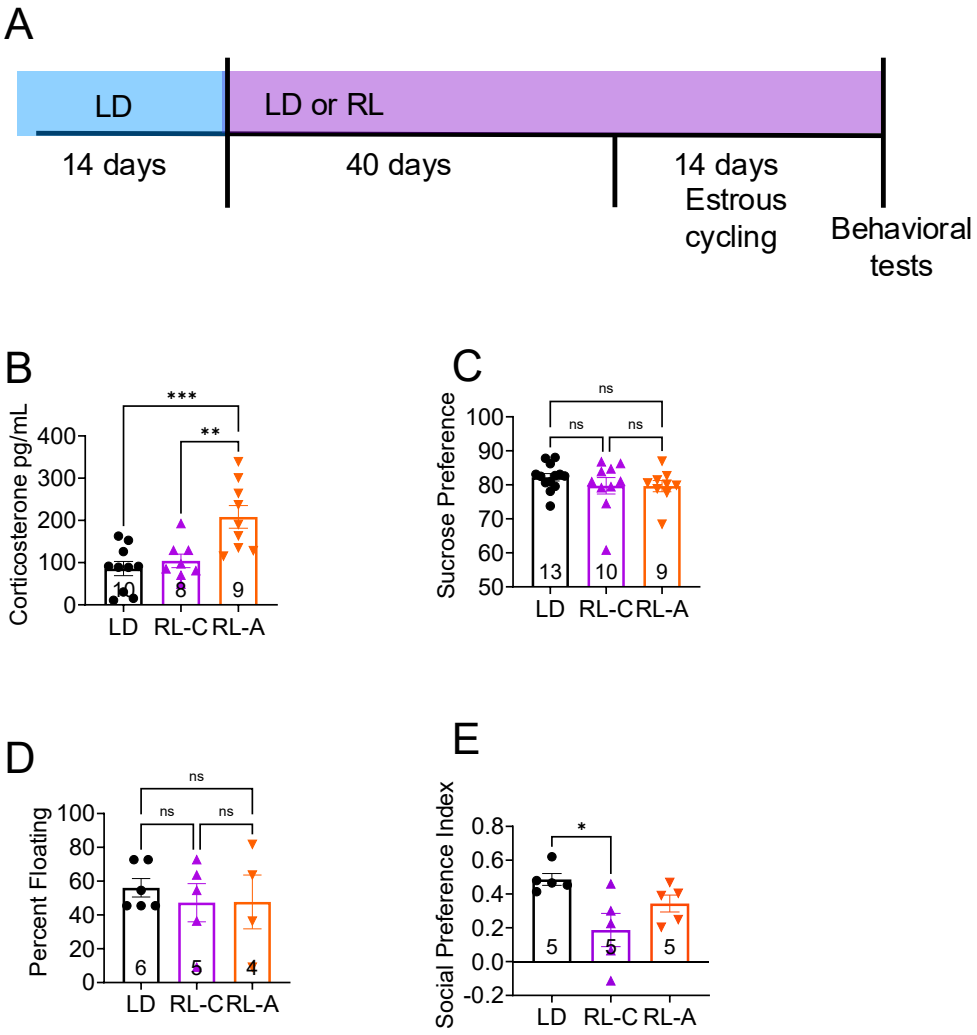

Figure 4.6. RL-A mice have increased circulating corticosterone, but no changes in depressive-like behaviors. A) An experimental timeline where blue indicates all mice in stable LD lighting conditions while purple indicates experimental mice in RL and controls in stable LD lighting. B) Terminal corticosterone from the serum of female LD, RL-C, and RL-A mice 3-4 days following a phase advance in metestrus at ZT3, collected in a cohort that did not undergo the behavioral experimental timeline. One-way ANOVA. ** $p < 0.01$; *** $p < 0.001$. C) Sucrose preference of LD, RL-C, and RL-A mice at ZT13 during metestrus. D) Percentage floating in the Porsolt forced swim test in LD, RL-C, and RL-A mice at ZT13 during metestrus. E) Social preference index of LD, RL-C, and RL-A mice at ZT13 during metestrus. One-way ANOVA. * $p < 0.05$.

Independent of cyclicity, female mice exhibit normal fertility

Appropriately high levels of ovarian progesterone are key for successful pregnancy establishment and maintenance [314], [315]. To determine if exposure to RL impacts the ability of females to achieve and maintain pregnancy, we conducted a fertility test. After the initial 9 weeks in the LD or RL paradigms, an experienced control male was introduced to each cage for two weeks with daily plug checks. Pups were counted on gestational day 14 (GD14), defined as 14 days after the identification of a copulatory plug, or at term pregnancy, during labor (Fig. 4.7A). Compared to LD mice, RL mice did not experience any reduction in fertility as exhibited by the percentage of pregnant mice after two weeks of being co-housed with a male (One Way ANOVA, $p > 0.05$; Fig. 4.7B). In contrast, there was an overall significant reduction in pup numbers in RL mice compared to LD mice (Fig. 4.7C) (Two-way ANOVA, $p = 0.048$).

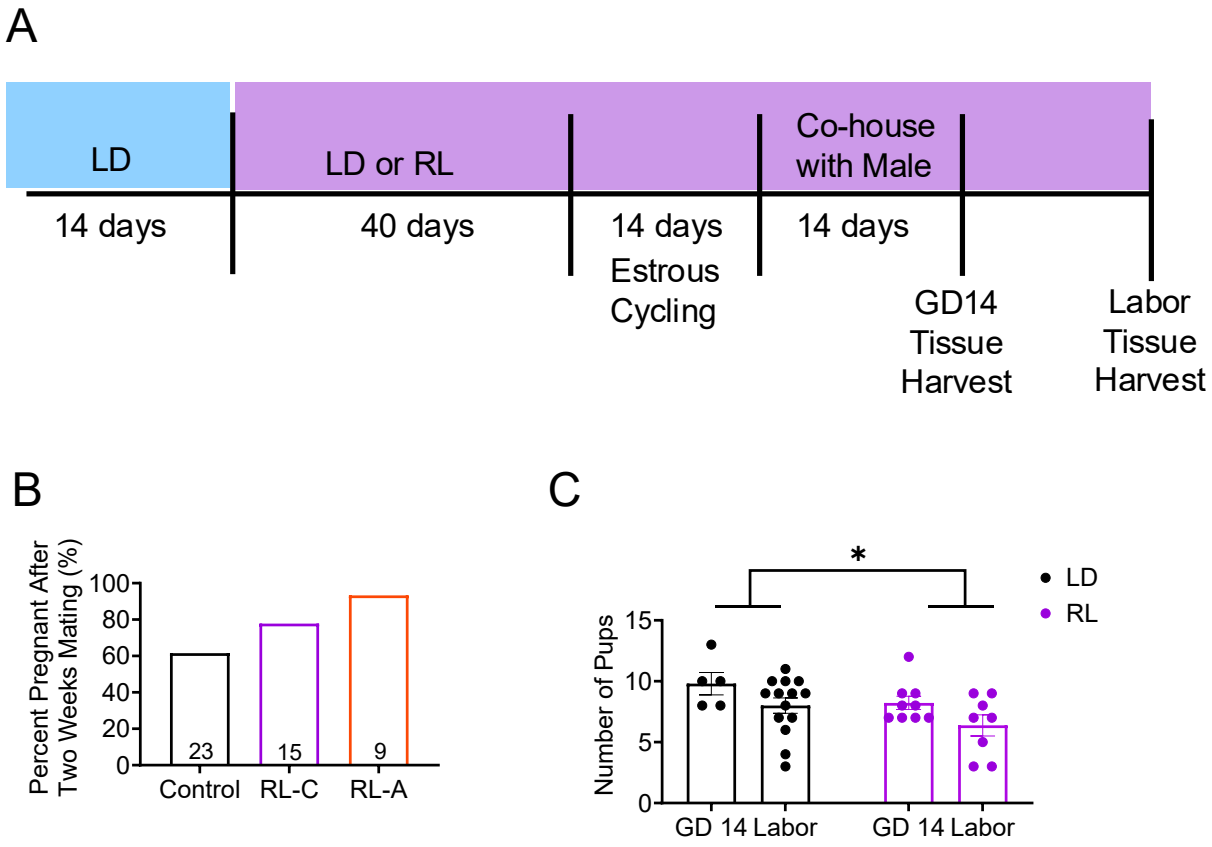

Figure 4.7. Both RL mice have smaller litters than LD females. A) Experimental timeline of study. B) The percent of mice that were pregnant after 2 weeks of co-housing with a control male mouse. C) Number of pups in the uterus at gestational day (GD) 14 and at labor in LD and RL mice. Two-way ANOVA, *, $p < 0.05$.

Discussion

Shift work negatively impacts the reproductive health of some, but not all, women, through the physiological differences that make some susceptible to this effect and others resilient are unknown. In this study we confirm that RL leads to a dichotomic phenotype of estrous cyclicity, with 50% of females exhibiting abnormal estrous cycles (RL-A), while the other 50% remain cyclic (RL-C). Physiological differences were identified between these two groups, where RL-A mice display mistimed rhythms in the SCN, ovary, and uterus. RL-A mice also show a reduced LH response to exogenous GnRH, an increase in atretic follicles in the ovary, and a reduction in circulating progesterone. Despite elevated corticosterone and low progesterone, RL-A mice did not exhibit increased depressive-like behaviors compared to LD or RL-C mice. Interestingly, both RL-A and RL-C were able to become pregnant in the 2-week mating period to a comparable level as LD mice, although RL females had smaller litters. This indicates that developing abnormal estrous cycles in response to rotating lights does not significantly reduce the capacity to conceive, but does reduce litter size.

Rotating shifts have a dichotomic effect on female mouse estrous cycles

As industrialization and demands for 24 h services rise, more people participate in shift work to meet the growing need. These shifts vary in length, duration, and shift size, yet all negatively impact female reproductive health [6]. Interestingly, not all women develop menstrual issues when participating in shift work, even when working the same shifts as women who do develop reproductive health deficits [285], [290]. Previous work using mouse models have similarly observed a dichotomic phenotype in cycle regularity in female C57B/6 mice. When exposed to rotating lights with a 10 hour shift every 3-4 days for 9 months, Bahougne et al. found that 4 out of 9 female C57B/6 mice demonstrated irregular estrous cycles [56]. Similarly, Yoshinaka et al. showed that a full 12 hour light inversion every 3 or 12 days for 36 days results in 12.5% and 25% of female C57B/6 mice failing to complete a full estrous cycle respectively, with these effects lasting up to three weeks following the return to normal LD lighting [297]. Both groups found a dichotomic phenotype in estrous cyclicity, where some mice retained estrous cycles comparable to controls, while other mice developed irregular estrous cycles. Our findings confirm this dichotomic

phenotype using a rotating light paradigm alternating between 6 h delays and 6 h advances every 4 days for 6-9 weeks. Importantly, while our study was conducted using different phase shift sizes and durations of shifts from previously published work [56], [297], in each study estrous cycles were negatively impacted, indicating a consistent and robust negative impact on estrous cyclicity in response to irregular light:dark cycles.

The female mouse SCN does not exhibit a dichotomic phenotype in response to rotating shifts

As the primary site of light translation into neural signals that regulate physiology, the SCN is a logical area of interest for potential differences between mice that are susceptible to rotating lights and those that are resilient. However, our data show that there are no differences between RL-A and RL-C mice in circadian SCN output or SCN PER2::LUC rhythms. Wheel running is a frequently used proxy for SCN circadian output [316], [317] and instead of finding differences between RL-A and RL-C mice in wheel running behavior, we instead found that both groups failed to entrain behaviorally to phase advances compared to phase delays. Previous work supports this finding, as mice do not entrain as well to phase advances [318]–[320]. This failure to entrain to advances was further confirmed with our PER2::LUC data, which shows that the SCN from RL-C (advance) mice has a significantly different phase than the SCN from LD mice, indicating a failure to entrain to advances. However, the SCN from RL-C (delay) mice are comparable to the SCN from LD mice in phase, supporting the claim that female mice fail to entrain to phase advances, but successfully entrain to phase delays. However, as no significant differences were found behaviorally between the RL-A and RL-C mice, investigation into the rest of the HPG axis to determine the source of the dichotomic phenotype was required.

The pituitary gland of female mice exposed to rotating light shifts exhibits a dichotomic phenotype in function but not circadian rhythms

Female reproductive health is highly dependent on the proper function and hormone release from the pituitary gland [85]. Our work suggests dysfunction at the level of the pituitary gland in RL-A mice in functional response to GnRH despite normal circadian function, when recording from the intact pituitary. In our PER2::LUC recordings, both RL-C and RL-A mice fail to cluster in the phase (time of first peak)

when collected during advances, but show significant clustering when collected during delays. If the pituitary gland were to entrain to these delays, we would observe a peak phase about 6 h after the LD pituitary, which is what we observe, indicating that the pituitary glands of RL-C and RL-A mice do not differ in circadian rhythms. Previous work supports this, with pituitary glands entraining quickly to phase changes [321] and maintaining a robust circadian rhythm [46]. Functionally, we found that the pituitary glands of RL-A mice are less sensitive to exogenous GnRH than the pituitary glands of LD and RL-C mice, indicating a reduction in function of the pituitary gland that may limit the LH response to GnRH. Previous work with the full body *Bmal1* knock-out mice shows no change in sensitivity to GnRH, but rather an increased sensitivity to kisspeptin [98], supporting the idea that this difference in response to GnRH is potentially unrelated to the function of the molecular clock within the pituitary gland. We also attempted to measure the response to kisspeptin, but due to equipment malfunction, the data were unusable, and the samples were lost. It may instead be that the release of endogenous GnRH is mistimed, resulting in changes in pituitary sensitivity to GnRH release [309] or that the expression of GnRH receptor in the pituitary is downregulated [322]. These differences in pituitary function may negatively impact the ovary, as proper LH and FSH release is required for follicular growth and development in the ovary as well as ovulation [109]. Future work could attempt to examine the pulsatile levels of LH and FSH using serial blood collections to determine if reduced LH or FSH is the driving cause behind the differences in ovarian health and progesterone release in RL-A mice compared to LD and RL-C mice, though the high levels of corticosterone in the RL-A mice may limit this technique as high corticosterone levels prevent the LH surge [243], [323] and suppresses LH pulsatility [324].

The ovaries of female mice exhibit a dichotomic phenotype of altered rhythms, follicle counts, and hormone release in response to rotating light shifts

An important metric for female reproductive health is ovarian health [325], [326]. The ovary displays circadian rhythms closely related to follicular development and reproductive function. The circadian rhythms of the ovary are well documented [327], [328], as well as in specific ovarian cell types including theca cells, granulosa cells, and oocytes [104]. Though our data show a comparable count in corpus luteum

in LD, RL-C, and RL-A ovaries, RL-A mice have a significant decrease in circulating progesterone compared to LD and RL-C mice. This suggests that, while the number of corpus lutea is not different, the function may be, which includes progesterone production. This is referred to as corpus luteal insufficiency and can lead to a luteal phase deficit, which often results in reduced fertility [329]. The corpus luteum relies on the pulsatile release of LH from the pituitary for maintenance and function [330], [331]. Corpus luteal insufficiency is thought to occur for one of three reasons: 1) the pulsatile release of LH from the pituitary is disrupted or insufficient [332]. 2) The corpus luteum does not respond to LH [332], and 3) that an insufficient corpus luteum can form from a substandard preovulatory follicle, which can occur due to the suboptimal growth of the follicle itself or the LH surge occurring too early in follicular development [333]. It is reasonable to think that our model may experience the LH surge too early in follicular development due to the pituitary entraining to the phase shifts while the ovary lags behind. Another possibility is that the reduction in progesterone is due to a disruption at the level of the ovary itself. Prior work has shown that intact circadian rhythms and specifically BMAL1, a key molecular clock gene, are required for progesterone synthesis [334]. This has been demonstrated using genetic mouse models where the disruption of the molecular clock through the conditional deletion of *Bmal1* from rat granulosa cells or the steroidogenic cells reduces the production of progesterone [335], [336]. Full body *Bmal1* knock out models have been used to demonstrate that this circadian control of progesterone synthesis is due to the transcriptional effect of BMAL1 on steroidogenic acute regulatory protein (StAR), which is required for progesterone synthesis [337]. Therefore, the change in progesterone in the RL-A mice compared to the LD mice may be due to a disruption to the genes driving circadian rhythms as opposed to tissue level rhythms. One limitation of our methodology is the use of the full ovary for PER2::LUC recordings. It is possible that rhythms are disrupted in specific cell types in the RL-A mice compared to both the LD and RL-C mice, however our current methodology would be unable to detect those differences as whole tissue recordings measure all cell types in contact with the MilliCell membrane. Future work could determine cell-specific circadian dysregulation in the ovary using single cell sequencing to determine the expression of clock genes at various time points in specific cell populations of the ovaries. In addition to the potential reduction in

corpus luteum function, our study finds that mice that develop irregular estrous cycles in response to RL have an increase in atretic follicles, indicating higher follicular death, than both LD and RL-C mice. The significant decrease of secondary and pre-antral follicles in the RL-A mice also indicates a decrease in developing follicles. Previous work has found that follicular health and development is reliant on the pulsatile release of LH and FSH from the pituitary [85]. Studies focused on polycystic ovarian syndrome (PCOS) have found that decreasing LH as well as altering the LH/FSH ratio, can increase the presence of atretic follicles and decrease the presence of maturing follicles [338]. While no significant differences were found in LH and FSH at baseline (FSH data not shown) in LD, RL-C, and RL-A, these collections were taken at a single time point and do not account for FSH's and LH's pulsatile nature. Future experiments could attempt to rescue ovarian function using GnRH pumps to regulate the LH and FSH release from the pituitary in RL-A mice. If successful, this would suggest the pituitary gland to ovary relationship as the difference between those who develop irregular estrous cycles in response to rotating lights and those who retain normal estrous cycles.

Rotating lights do not increase depressive-like behaviors in female mice

Circadian disruption has been shown to have a significant negative impact on mental health and mood disorders in humans [55], [339]–[341]. Previous work with shift work-like lighting and depressive-like behaviors in male rodents has found that chronic exposure to an advancing light schedule results in an increase in depressive like behavior [342]. Similarly, in male rats, rotating shifts result in an increase in depressive-like behaviors as well as anxiety-like behaviors [343]. Surprisingly, we did not detect an increase in depressive-like behaviors in either of the RL groups. This is particularly surprising in the RL-A group, which had lower levels of progesterone, and high corticosterone, two knowns risk factor associated with depressive disorders in women [191], [260], [344]–[346]. It is unclear why the RL-C mice appear to experience a decrease in social preference, as evidenced by less time spent in the chamber with the novel female mouse. However, due to the relatively low number of animals included in the study (n=5 per group), it is possible that our data are a false positive [301], [347], [348]. Further work is needed to confirm the validity of our results and determine potential mechanisms by which they are arising.

Female fertility remains intact in mice exposed to rotating lights independent of cyclicity

In addition to the negative impacts on the menstrual cycle, shift work also has negative impacts on fertility and pregnancy, specifically rotating shifts can reduce female fertility, increase the likelihood of miscarriage, and reduce birthweight [6]. While we found mistimed rhythms in uterine samples in non-pregnant mice exposed to rotating lights, we also found that fertility is comparable to LD mice in both RL-A and RL-C mice, suggesting that the mistiming was not significant enough to impact fertility when allowing 14 days of male-female cohousing for pregnancy to be established. However, uterine rhythms are important for implantation [283], [349] and the mistimed PER2::LUC rhythms in both RL-C and RL-A mice we observed may have a negative impact on implantation and be the cause of the reduction we see in pup numbers in RL mice. Our work also suggests that the infertility associated with shift work is may have a different cause than shift work-induced irregular menstrual cycles. This idea is supported by studies examining women who experience regular menstrual cycles, yet struggle with infertility [350], [351], and women who experience painful or irregular cycles without infertility issues [352], [353]. While our work did not see a difference in the percent of mice that successfully conceived in two weeks, it is possible that mice exposed to rotating lights may take longer to conceive. Our experimental design used co-housing as opposed to timed mating to focus on the possibility of the mice becoming pregnant and therefore does not allow us to answer that question, but future work could use the more precise timed mating technique to determine if the timing of conception is impacted.

Conclusion

The field of female reproductive health beyond exclusively examining fertility is steadily emerging. This work identifies that rotating light shifts result in irregular estrous cycles in some, but not all, female mice, examines desynchrony, and function or morphology at each level of the HPG axis, and supports the negative impacts of rotating lights on ovarian function and reduced follicle quantity, in some but not all female mice. Together, this work expands our understanding of the negative impacts of rotating lights on female reproductive health.

Acknowledgements: We would like to thank both the Robison Lab and the Veenema Lab at Michigan State University for the use of behavioral equipment and training.

CHAPTER 5: DISCUSSION

Shift work is detrimental to many facets of female reproductive health, extending beyond just fertility but also affecting menstrual health, reproductive hormones, and pregnancy [6]. To better understand how the circadian disruption that occurs during shift work impacts reproductive health, we must first understand the regulation of the suprachiasmatic nucleus (SCN) of the hypothalamus, which is the pacemaker and synchronizer of circadian rhythms [64], [267], [354]. The goal of this dissertation was to determine how the regulation of the SCN by homeodomain transcription factors or mistimed light exposure impacts circadian rhythms, fertility, and estrous cycles in a mouse model, with a primary focus on females.

Summary of findings

These studies collectively demonstrate that precise molecular and neuronal function within the suprachiasmatic nucleus (SCN) is essential for regulating circadian rhythms, reproductive health, and mood, particularly in females. Genetic manipulations targeting SCN-enriched transcription factors revealed distinct roles for *Vax1*, *Six3*, and *Six6*. Deletion of *Six3* in NMS-expressing neurons shortened circadian locomotor rhythms in males and females, but only impacted the male reproductive axis, as evidenced by reduced sperm motility in males, while *Six6* deletion had no significant effect on any of the measured outcomes. Similarly, Deletion of *Vax1* in VIP-expressing SCN neurons weakened circadian output, disrupted female estrous cyclicity and increased the sensitivity of the GnRH neurons to Kisspeptin, and led to increased depressive-like behavior, without affecting male or female fertility. These findings suggest *Vax1* and *Six6* maintain critical post-developmental functions in specific neuron populations of the SCN. Together, they underscore how molecular disruptions within the SCN can cascade into broader physiological and behavioral dysfunctions. Both SIX3 and VAX1 regulate the expression of genes involved in the molecular clock. To determine if mistimed light, another regulator of the molecular clock, would have similar impacts on female reproduction, we examined how chronic exposure to rotating light cycles which mimic human shift work, impacts female mice. Half of the mice exposed to rotating lights (RL) developed irregular estrous cycles, while the other half remained resilient, reflecting a dichotomous

physiological response. Mice with disrupted cycles also showed impaired levels of progesterone and a reduction in developing follicles in the ovary. These outcomes parallel those observed in human shift workers, where only some individuals experience reproductive health issues, suggesting an underlying biological vulnerability. By identifying distinct behavioral, hormonal, and molecular signatures between resilient and susceptible mice, this work provides a critical bridge between genetic vulnerability, environmental stressors, and reproductive health outcomes, emphasizing the SCN's central role as a regulator of circadian-reproductive integration. Together this work shows that deregulation of the SCN, through either conditional deletion of homeodomain transcription factors or through environmental lighting changes, can negatively impact female reproductive health.

Discussion

Identification of novel roles of *Six3* and *Vax1* in the post-developmental mouse SCN

Six3 and Vax1 are involved in circadian regulation of the adult mouse SCN

Light and other environmental factors regulate the SCN through transcriptional regulation [30]. Therefore, understanding the transcriptional regulation of the SCN is important to better understand circadian rhythms and their downstream effects on reproduction. Most of the work to date on the transcription factors enriched in the SCN, including *Six3*, *Six6*, and *Vax1*, explores on their roles in development [122], [125], [126], [355], [356]. These three transcription factors are required for the proper development of the SCN, however, they remain expressed in the adult SCN where their functions remain underexplored [67]. Previous studies have used full-body heterozygous knockouts [120], [199], [355], Synapsin-cre which is expressed in all/most mature neurons [131], or target neuron populations that are not exclusively in SCN [131], [357], limiting their ability to determine the roles of transcription factors in the adult SCN with specificity. With the goal to target the adult SCN, the work in this dissertation uses models which have overlapping expressing of the Cre-allele and the Flox-allele close to exclusively in the SCN. Specifically, we combined SCN neuropeptide enriched Cre-alleles that turn on after SCN cell proliferation, in conjunction with the three transcription factors studied, all which are highly expressed in the SCN. This work finds that *Six3* and *Vax1* play important roles in the regulation of circadian rhythms, specifically

within SCN neurons that express VIP, and that *Vax1* in VIP neurons mediates female reproductive health. These findings expand our understanding of the roles of homeodomain transcription factors and their functions in the adult SCN in a non-invasive and relatively cost-effective way.

Though this work finds that *Six3* and *Vax1,* but not *Six6,* regulate circadian rhythms in the adult mouse SCN, the mechanism behind this process remains unclear. As these effects are seen with the loss of *Vax1* or *Six3* from VIP neurons, a reasonable hypothesis may be that they impact the expression of VIP peptide, in turn shortening rhythms, as full body VIP knock out models exhibit similar shortened rhythms to our mouse models [77]. However, SIX3 does not regulate VIP expression [121], and, while VAX1 can regulate VIP expression [121], the difference between *Vip* mRNA in our $Vax1^{VIP}$ conditional knock out mice was insignificant, suggesting that the alteration in free-running period exhibited in both $Six3^{VIP}$ and $Vax1^{VIP}$ mice was accomplished using a different mechanism. One potential mechanism may involve alterations in the expression of Gamma-aminobutyric acid (GABA). Nearly all VIP neurons in the SCN express GABA, a small often inhibitory, neurotransmitter [67], [358] that functions in the SCN to synchronize and refine cellular circadian rhythms [359]. Though limited, previous studies have found that loss of *Vax1* early in development impairs GABA neuron development [360] and SIX3 is enriched in GABA neurons in the substantia nigra [361]. These current studies provide basic evidence for both SIX3 and VAX1 expression within GABAergic neurons, but future work will be required to determine any relationships between GABA and these homeodomain transcription factors as well as the exact mechanism through which SIX3 and VAX1 impact VIP neuron function within the SCN. Another potential mechanism for this change in free-running period may be that SIX3 and VAX1 regulate VIP neuron function through their regulation of the molecular clock. Both VAX1 and SIX3 have been shown to regulate the core genes of the molecular clock with VAX1 upregulating PER2 [121] and BMAL1 (Chapter 4) and SIX3 upregulating PER2 [121]. Interestingly, we also found that SIX6 upregulates BMAL1 and PER2 (Chapter 2), yet we see no change in the circadian phenotype of $Six6^{NMS}$ mice. This may point to the compensatory role of Six3 upon loss of Six6, which has been previously found [355]. If loss of *Vax1* or *Six3* alters clock gene expression, then the timing of VIP release may be changed, resulting in a shortened free-running

period. To determine if this is the case, a time course study would be required, taking samples from mice across a 24 hour period, to measure the rhythm of SCN VIP expression.

Vax1 in VIP neurons of the SCN plays a role in regulating estrous cycles in the female mouse

The homeodomain transcription factors SIX3, SIX6, and VAX1 have been implicated in the regulation of female reproductive health [120], [126], [131], [199], but studies to date have been unable to isolate if this function is related to their role in development or their function in the adult brain. Using conditional knock out models, this work finds that *Vax1,* but not *Six3* or *Six6*, in the adult SCN plays an important role in female reproductive health. Previous work has found reproductive deficits in mice that have lost *Six3* [126], [131], *Six6* [120], or *Vax1* [199]. However, these studies were limited by the use of heterozygous full body knock outs prior to development, or non-specific deletion using Synapsin-cre to target maturing neurons throughout the entire nervous system. Our studies utilize Cre-alleles that target neurons that express neuropeptides expressed in the SCN post development, allowing us to determine the roles of these homeodomain transcription factors later in the developmental process. Using post neuronal proliferation conditional KO mice, we found that loss of *Six3* or *Six6* from NMS neurons did not impact female reproduction, indicating that the known roles of *Six3* and *Six6* in female reproduction are likely due to their role in development as opposed to their function in the adult SCN. *Vax1*, however, when deleted from VIP neurons, did in fact negatively impact female, but not male, reproductive health by lengthening estrous cycles and reducing the concentration of circulating estrogen, a similar phenotype to the full body VIP knock out mouse [77]. This indicates that at least a part of the role of *Vax1* in the VIP neurons of the SCN is the regulation of the female estrous cycle and hormone levels. Interestingly, $Vax1^{VIP}$ female mice did not exhibit the same level of reproductive deficits as *Vax1* Synapsin-cre mice [121], which may suggest that VAX1 expressed outside of the SCN also plays a role in the regulation of female fertility while the function of VAX1 within VIP neurons of the SCN is more strongly related to the estrous cycle than fertility. It would be of interest in future work to determine the extra-SCN brain structures that express SIX3, SIX6, or VAX1 and are required for female fertility.

No sex differences in the transcriptional regulation of reproductive or circadian processes

Many aspects of circadian rhythms are sex-specific, from timing of neuropeptide expression to the morphology of the SCN itself [215]. Our findings show that the roles of SIX6, SIX3, and VAX1 are not sex specific in reproduction or circadian regulation. No significant circadian or reproductive phenotypes were found in $Six6^{NMS}$ male or female mice. No reproductive phenotypes were found in $Six3^{NMS}$ mice with the exception of a reduction in male sperm motility, though we hypothesize that this is due to ectopic Cre expression in the testes as opposed to a downstream effect of SCN *Six3* deletion (see "Limits of this Dissertation" below). Similarly, in both male and female $Vax1^{VIP}$ mice, we see reductions in markers of reproductive health, such as later puberty onset in males and longer estrous cycles in females, but we see no functional difference in fertility, as both male and female $Vax1^{VIP}$ mice were comparable to controls in time to first litter and the number of litters in 90 days. These results contrast previous results with the full body VIP knock out models, which result in sub fertile females [77], though male VIP knock out mice appear to have increased protection against testicular aging, a change that is likely to increase fertility rather than decrease it [175]. It may be that, while VIP peptide plays an important role in female, but not male, reproduction, *Vax1* within VIP neurons plays a modulatory role of both male and female reproduction. The importance of transcription factors in SCN, and specifically VIP, neurons for both sexes is further supported by our findings that the functions of SIX3 and VAX1 in the regulation of circadian rhythms are also not sex specific. This was surprising, as VIP expression and the rhythmicity of its release are sex-specific in both humans and animals with men having higher numbers of VIP cells in the SCN [216] and sexually dimorphic rhythms apparent in animal mRNA [362]. We similarly found an increase in VIP expression the male SCN of mice compared to the female SCN (Chapter 3). Due to this, we expected that any alterations to VIP-expressing neurons would have a sex-specific effect. However, we see that both male and female mice with a loss of *Six3* or *Vax1* experience a shortening in circadian period to a comparable degree. This suggests that whichever mechanism is being affected is held in common between the sexes.

Mistimed photic cues regulate the SCN and result in an increased risk for irregular estrous cycles

The female mouse SCN fails to adjust to phase advances but adapts to phase delays

Due to the increase in industrialization and demand for services at all times of day, humans work a wide variety of shifts that alter in length, duration, and shift size [6], [363]. The health impacts of these shifts vary in humans, with some having minimal effects on human health [364]–[366], while others are detrimental to cardiovascular, gastrointestinal, and reproductive health [367]–[372]. Previous work in rodents has also used a variety of light shift paradigms, from 10 hour shifts alternating between advances and delays every 3-4 days for 9 months [56], to complete inversions (12 hour shifts) every 3 days [297]. Others have used chronic advances [319], [320] or chronic delays [373], [374], though in humans, shifts rarely move in a singular direction [375]. To determine if our lighting paradigm, which was designed with 6 hour shifts every 4 days in an attempt to mirror human shift cycles, would regulate the SCN and have the potential to impact health, we measured the entrainment of the SCN to the rotating lights (RL) paradigm. Wheel running is a frequently used proxy for SCN circadian output [316], [317] where we found that mice exposed to RL fail to entrain behaviorally to phase advances compared to phase delays. This failure to entrain to advances was further confirmed with our PER2::LUC data, which shows that the SCN from RL mice collected during a phase advance has a significantly different phase than the SCN from LD mice, indicating a failure to entrain to advances. However, the SCN from RL collected during phase delays mice are comparable to the SCN from LD mice in phase, supporting the claim that female mice fail to entrain to phase advances, but successfully entrain to phase delays. Previous work supports this finding, as mice do not entrain as well to phase advances [318]–[320]. This failure to adjust to phase advances suggests that our model is sufficient to study the impacts of shift work-like lighting on reproductive health while maintaining a comparable shift size and frequency to human shift work.

Mistimed photic cues during RL negatively impact estrous cycles in half of female mice

Rotating lights have been found to have detrimental effects on reproductive health in humans [6], [115], [284]. Similarly, previous work using mouse models has shown that chronic exposure to rotating light shifts can disrupt the estrous cycle in around 50% of mice [56], [297]. One such study used chronic

10 hour shifts, rotating between a delay and advance every 3-4 days for 9 months resulted in approximately 50% of their mice becoming acyclic [56]. Another study found that a complete inversion of the lights (a 12 hour shift) every 3 or 12 days similarly resulted in a sub-set of mice with an irregular estrous cycles [297]. Similar to these studies, our work finds that when exposed to 6 hour shifts rotating between advances and delays every 4 days for 9 weeks results in 50% of the female mice developing irregular estrous cycles. This indicates that rotating light shifts are likely to trigger the loss of estrous cyclicity in roughly half of the mice exposed. This is supported in human studies, where not all female shift workers experience menstrual cycle issues or infertility [285], [290], [291], but rather, a subset. It is likely, then, that there may be biomarkers in the HPG axis that may indicate which individuals will develop reproductive issues in response to shift work-like lighting and which will not. Future studies should examine potential biomarkers in mice prior to exposure to rotating lights to find predictive measures that could potentially be used to identify humans that may be susceptible to shift work.

Dysregulation of the SCN can negatively impact female reproductive health without negatively impacting female conception rates.

Disrupted circadian rhythms have been found to negatively impact female fertility [6], but also have detrimental effects on overall reproductive health including the menstrual cycle [285], [290], [291], hormonal health [115], [376]–[378], and risk of breast cancer [376], [379], [380]. Our work finds that both loss of *Vax1* from SCN VIP neurons and exposure to rotating lights can negatively impact female reproductive health in the context of estrous cycles, sex steroid hormones, and neural sensitivity to GnRH or Kisspeptin within the hypothalamus without having an impact on conception. Previous work in humans shows irregular menstrual cycles or hormonal release in shift working women [285], [289], [291], but to date studies have not determined if these women experience infertility as a result of these irregular cycles. Future studies in humans should be used to determine if SCN dysregulation can regulate both menstrual health and fertility separately, as our work indicates that the SCN regulates female reproductive health in more ways than fertility alone and the proper regulation of the SCN is required for female wellbeing.

SCN dysregulation results in sexually dimorphic depressive-like behaviors in mice

Circadian disruption has been shown to have a significant negative impact on mental health and mood disorders in humans [55], [339]–[341]. Previous work with shift work-like lighting and depressive-like behaviors in male rodents has found that chronic exposure to an advancing light schedule results in an increase in depressive like behavior [342]. Similarly, in male rats, rotating shifts result in an increase in depressive-like behaviors as well as anxiety-like behaviors [343]. Therefore, when we disrupted circadian rhythms in our female mice using a rotating light paradigm, we expected to see an increase in depressive-like behaviors, especially in the RL-A group as they have lower levels of progesterone, and increase in corticosterone, which have been associated with depressive disorders in women [191], [260], [344], [345]. Surprisingly we did not see an increase in depressive-like behaviors using three different tests. Instead, there was no difference between RL-A mice, RL-C mice, and controls. It is possible that changing the size of the shifts or the duration may change these results, with larger shifts leading to the expected phenotype. It is probable that there is a sex difference in the process that gives rise to depressive-like symptoms in response to chronic shift work-like lighting. We see such a sex difference when examining the depressive-like behaviors of our $Vax1^{VIP}$ male and female mice. We saw an increase in depressive-like behaviors in the female, but not male, mice, again suggesting a sex difference in the process. It is possible that the sex difference is sex steroid hormone related as the relationships between female reproductive hormones and mood disorders is well established and reviewed, with low levels of progesterone as well as both high and low ratios of progesterone to estrogen correlating with an increase in depressive symptoms [257], [381]–[383]. It is also possible that the sex difference lies in the VIP neuron population or other neuron populations within the SCN as the SCN is itself sexually dimorphic [215]. While more work will be required to determine the exact mechanism through which VAX1 in the SCN is regulating female behavior, the knowledge that it may be involved in depressive-like behaviors give us a new mechanism to continue to work to treat and prevent depression, specifically among shift workers. Future work will be needed to determine the specific mechanisms that are driving this light shift induced depressive-like behavior and determine the differences between the sexes.

Limitations of the Dissertation

Along with the limitations already discussed in each individual chapter, there are a number of additional limitations of this dissertation as a whole. The first is the use of the murine model to mimic human physiology. This model was selected due to its abundance of available transgenic alleles that could be manipulated for our studies' purposes. However, it is important to acknowledge that, especially in circadian work, the use of mice limits the generalizability of the work to any human condition as mice are nocturnal and humans are diurnal. This limitation, however, does not limit the growth in knowledge. It just means that statements must be made with care and any discovery that may benefit humans must first be explored in a more biologically relevant model.

Another limitation is the use of the Cre-Recombinase/*LoxP* system. The use of this system limits the conditional loss of the homeodomain transcription factor in question only to the cells expressing Cre-recombinase, which may not encapsulate all of the target cells within the SCN. Another limitation of the Cre/*LoxP* system is the likelihood of off-target expression of Cre-recombinase. Previous work has found that Cre-recombinase expressing mice may express Cre-recombinase in cells other than the target cells [49] or the Cre-recombinase expression itself may give rise to a phenotype [48]. Our work does its best to mitigate this risk by using mice that have a heterozygous expression of Cre-recombinase along with using Cre+ Flox- mice and Flox/Flox mice as controls. However, limitations such as off-target expression or limited knock out success are still a consideration in these models.

Overall Conclusion

I hypothesized that dysregulation of the SCN through loss of transcription factors *Six3*, *Six6*, or *Vax1* or through mistimed light exposure, will negatively impact reproductive health in mice. Together, this work finds that dysregulation of the SCN through conditional loss of *Vax1* or through mistimed light exposure can result in negative impacts on female reproductive health including reduced sex steroid hormones and irregular estrous cycles with minimal effects on male reproductive health. Conditional loss of *Six3* results in a shortening of circadian period, but no noticeable effects on female reproduction. Conditional loss of *Six6* had no noticeable effects on circadian rhythms, or male and female reproduction.

Ultimately, the work of this dissertation finds that circadian regulation is important to reproductive health independent of its role in fertility.

It is important that work in both circadian regulation and female reproductive health continues. A majority of female reproductive health research is targeted towards solving the issues of infertility [283], [384]–[386]. While this goal is important, it is also important to explore other aspects of reproductive health that impact women's daily lives, including menstrual cycles, hormone imbalance, and menopause. The work discussed in this dissertation examines fertility but often finds that regulation of the reproductive axis can impact hormones, ovarian quality, and hormone cycles without impacting fertility itself. These findings are important to report in order to dissuade the belief that female reproductive health focuses only on fertility, but rather reproductive health should encompass the entirety of the process.

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