UNIVERSITY OF CALIFORNIA SAN DIEGO

Effect of Deletion of the Circadian Gene *Bmal1* in Neuromedin-S Neurons on Female Fertility

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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2020

The Thesis of Jinkwon Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2020

DEDICATION

I would like to dedicate my thesis to:

My parents for their ultimate love and support across the ocean.
My Korean, Argentine, and US friends for helping me assimilate to new cultures and broaden the scope of my thought.

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The thesis is coauthored with Mellon, Pamela and Tonsfeldt, Karen. The thesis author was the primary author of the thesis.

ABSTRACT OF THE THESIS

Effect of Deletion of the Circadian Gene *Bmal1* in Neuromedin-S Neurons on Female Fertility

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Master of Science in Biology

University of California San Diego, 2020

Professor Pamela Mellon, Chair
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The female reproductive system is coupled with circadian rhythms, and circadian disruption is associated with decreased reproductive capacity. For example, the preovulatory luteinizing hormone (LH) surge is temporally regulated. In the hypothalamus, the suprachiasmatic nucleus (SCN) sends projections to the kisspeptin and gonadotropin-releasing hormone (GnRH) neurons to properly synchronize their endogenous clocks.

Previous studies have demonstrated that the whole-body deletion of *Bmal1*, a critical molecular clock gene, in female mice results in no LH surge and infertility. However, the site of action of *Bmal1* in the LH surge is unknown. Recently, we demonstrated that mice with a conditional *Bmal1* knockout in either kisspeptin or GnRH neurons were fertile, with a normal LH surge. Therefore, we hypothesized that *Bmal1* in the SCN would be critical for female reproduction. Neuromedin-S (NMS) is a neuropeptide abundantly expressed in the SCN, and in nearly all VIP and AVP expressing neurons, which send projections to GnRH and kisspeptin neurons, respectively. *Bmal1* knockout in NMS neurons resulted in loss of behavioral circadian rhythm under constant darkness, verifying clock disruption in the SCN. Nevertheless, ovariectomized Bmal1$^{fl/fl}$-NMS^{Cre} mice showed an LH surge at the appropriate time when given exogenous estradiol, and with kisspeptin administration. They had normal estrous cyclicity and were fertile when mated with a wildtype male. Thus, despite Bmal1$^{fl/fl}$-NMS^{Cre} female mice showing arrhythmic behavior, they are fertile. However, preliminary results indicate that ovaries in constant darkness showed less numbers of corpora lutea meaning decreased ovulation. These data suggest that the reproductive neurons, the endogenous clock or SCN temporal regulation is sufficient for fertility.

INTRODUCTION

Female fertility is tightly regulated by both the hormones from the reproductive system and the timing cues from the circadian system. In females, the process of ovulation depends on the level of luteinizing hormone (LH) and the timing of the LH surge depends on the circadian time. The levels of LH are regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1) and the timing of the surge occurs at the end of the subjective night for humans and rodents (1,2). Both the reproductive and circadian systems are coupled together, contributing to the proper timing of ovulation. This characteristic is advantageous, as it allows the animals to release ovum at the time when they are awake and sexually receptive, thus increasing the success rate of fertility. However, the mechanism behind the coupling of these systems in controlling the reproduction is under ongoing study and any disruption in these systems can affect fertility.

The hormones along the reproductive system – The HPG axis

The key players in the mammalian reproductive system come from the HPG axis which is divided into three parts: the hypothalamus, the anterior pituitary gland, and the gonads (ovaries or testes) (Figure 1). In the hypothalamus, the kisspeptin (Kiss1) neurons in the arcuate nucleus (ARC) release kisspeptin onto the gonadotropin-releasing hormone (GnRH) neurons located mostly within the preoptic area (3). When the GnRH neurons are stimulated by the ARC Kiss1 neurons, they release GnRH onto the medial eminence in a pulsatile manner, stimulating the gonadotrope cells in the anterior pituitary (3). The gonadotrope cells then release both LH and follicle-stimulating hormone (FSH) into the bloodstream, mainly targeting the gonads for spermatogenesis and oocyte maturation and secretion of sex hormones, estradiol (E_2) or testosterone (4,5). These sex hormones then feedback to inhibit the activities of the ARC Kiss1 neurons and the gonadotropes in the

anterior pituitary, suppressing LH release (5). This characterizes a negative feedback mechanism in the axis. In females, there is an additional population of Kiss1 neurons in the anteroventral periventricular nucleus (AVPV), which are stimulated by high systemic levels of E_2 produced by mature follicles (6). This positive feedback initiates a surge of GnRH release onto the gonadotropes, which leads to a surge in LH release and ovulation (7,8). It is thought that the negative feedback on the ARC Kiss1 neurons is responsible for the pulsatile release of the GnRH, while the positive feedback on the AVPV Kiss1 neurons is responsible for the LH surge necessary for ovulation (9).

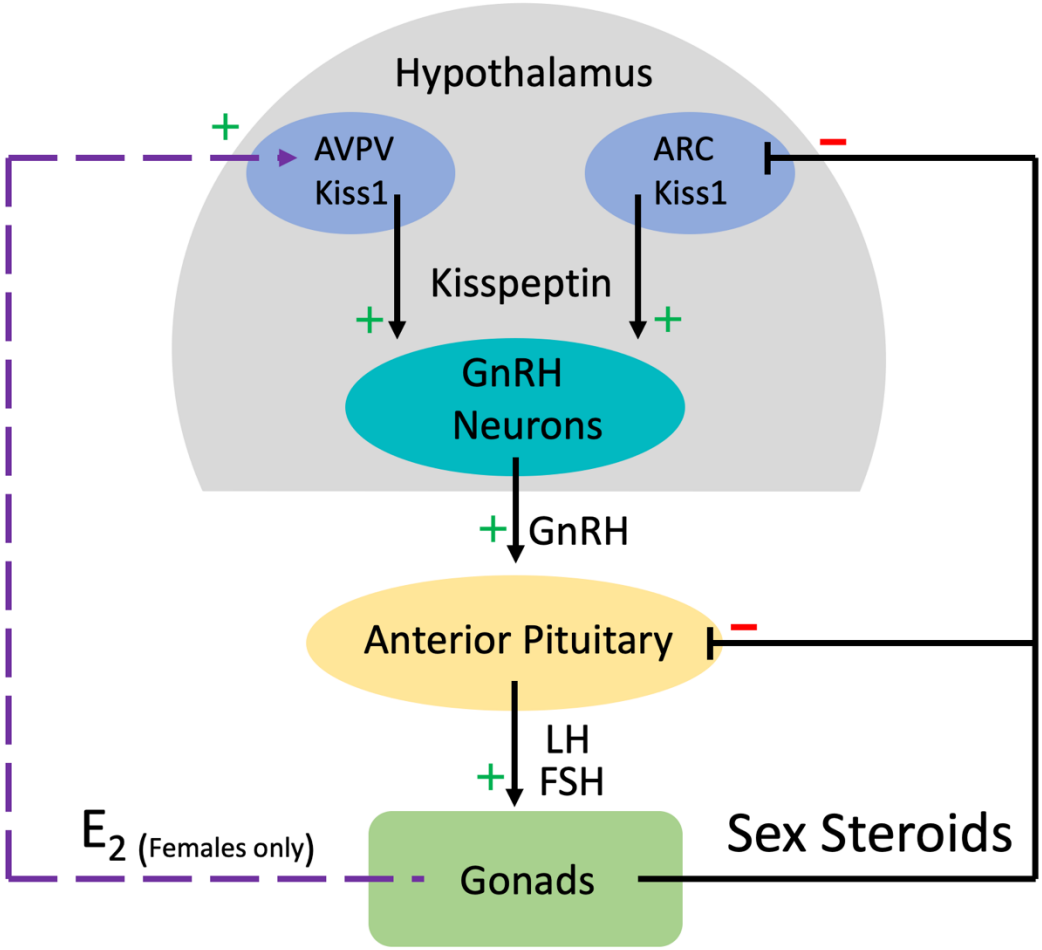

Figure 1. The hypothalamic-pituitary-gonadal axis regulates the reproductive system. In the hypothalamus, ARC Kiss1 neurons release kisspeptin to stimulate GnRH neurons. The stimulated GnRH neurons release GnRH to the anterior pituitary resulting in the release of LH and FSH into the bloodstream to target the gonads. Then, the gonads release sex steroids which provide negative feedback to the ARC Kiss1 neurons and to the anterior pituitary. In females, a high concentration of systemic estrogen stimulates the AVPV Kiss1 neurons, which then stimulate GnRH neurons, providing positive feedback to the HPG axis.

The molecular mechanism of the clock

The mammalian circadian clock functions as a negative feedback loop, best characterized as a transcriptional-translational feedback loop. The main proteins involved in this loop are two transcription factors, BMAL1 and CLOCK, and two families of repressor proteins, PER1-3 and CRY1-2 (Figure 2) (10). BMAL1 and CLOCK form a dimer that binds to E-box elements, promoting the transcription of the *Per* and *Cry* genes (11). When *Per* and *Cry* are translated and their protein levels have increased, they also dimerize and are translocated back to the nucleus. Once inside the nucleus, they repress the activities of the BMAL1/CLOCK dimers; the PER/CRY dimers inhibit the transcription of their own genes (11). Upon degradation of PER/CRY dimers, the BMAL1/CLOCK dimers are no longer repressed; *Per* and *Cry* genes are transcribed again, thus starting a new transcription-translation cycle which lasts approximately 24 hours (Figure 2). The circadian clocks are also present in most cells and peripheral tissues (12,13), but the suprachiasmatic nucleus (SCN) in the hypothalamus is the master-clock that synchronizes the clock elements of all over the body (10).

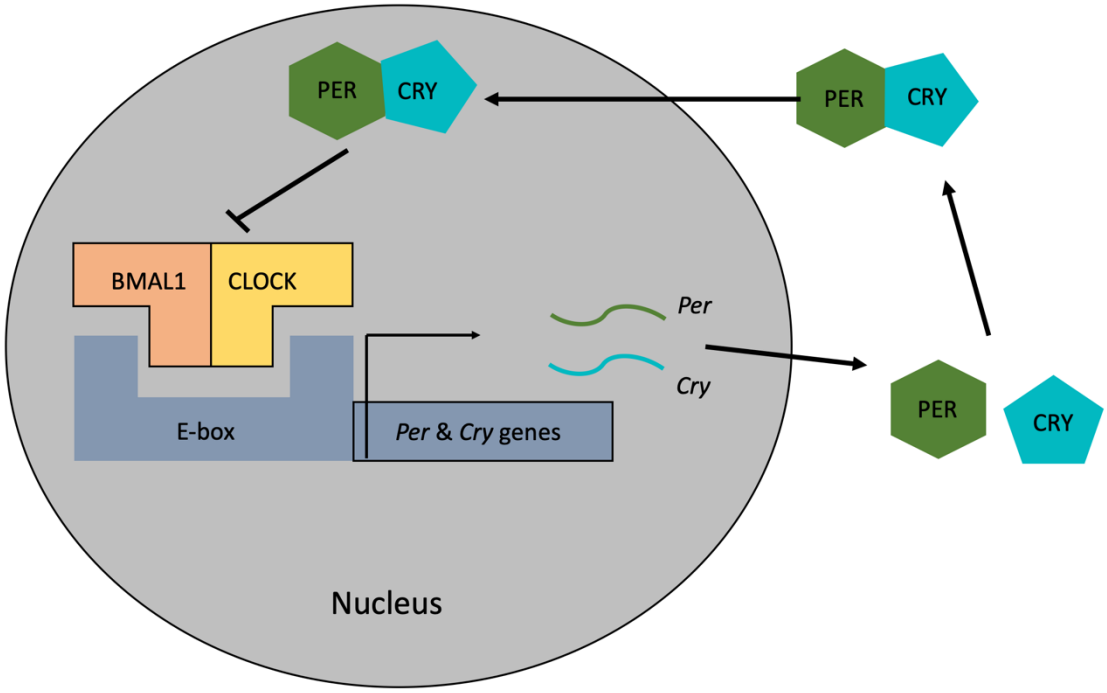

Figure 2. The molecular mechanism of the mammalian clock. BMAL1 and CLOCK form a heterodimer and bind to the E-box located in the promoter of the Per and Cry genes starting their transcription. When PER and CRY concentrations increase, they form a heterodimer and translocate to the nucleus. The PER/CRY dimer suppresses the binding of BMAL1/CLOCK dimer to the E-box inhibiting the transcription of their genes, and thus PER and CRY concentrations decrease. As PER/CRY dimers get degraded, the BMAL1/CLOCK dimers bind to E-box again, starting a new cycle that lasts for 24 hours.

The master clock of the body - the SCN

The SCN is a special bilateral locus in the hypothalamus located above the optic chiasm. It receives photic information from the retina through the retinohypothalamic tract (10). The SCN has outputs to many different brain regions including the preoptic area and the AVPV where GnRH and Kiss1 neurons are located, respectively (14). Moreover, because the SCN receives light information in mammals, it functions as the main pacemaker of the circadian system throughout the body. The SCN is divided into two subregions: the core and the shell (Figure 3). One distinguishing characteristic between the two regions is that in the core, most neurons express vasoactive intestinal polypeptide (VIP), and in the shell, most express arginine vasopressin (AVP) (10). Furthermore, it has been shown that the GnRH neurons are excited by VIP via VIP2R receptor (15,16) while the AVPV Kiss1 neurons are excited by AVP neurons via V1a receptor (Figure 3) (17,18). When the systemic estrogen levels are high, the AVPV Kiss1 neurons show increased activation in the afternoon suggesting a temporal regulation by the SCN (19). Treatment with a VIP antagonist delays and attenuates the LH surge (20), and VIP knockout mice are subfertile and have irregular estrous cycles. This neural network between the SCN and the HPG axis may explain how the SCN specifically regulates the appropriate time of ovulation. Furthermore, many lesion studies in the SCN blocked the LH surge, suggesting the importance of the SCN in producing the surge (22,23). However, this lesion could also have potentially lesioned the long GnRH axon projection from the preoptic area to the median eminence, as the SCN is located in between these areas. Overall, the maintenance of the rhythmic reproductive behavior in female mice is essential for successful reproduction.

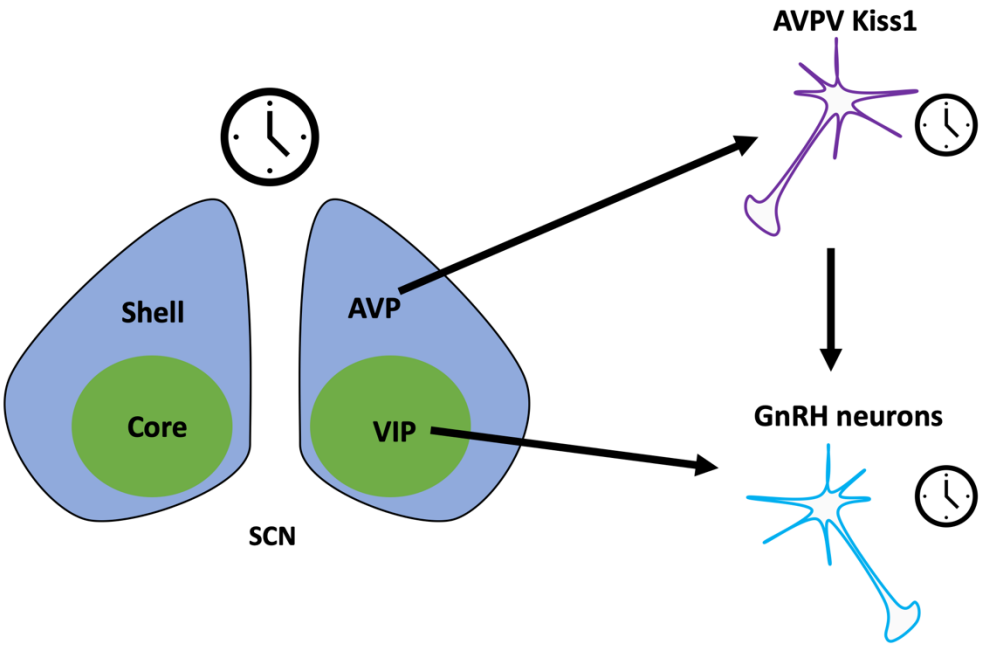

Figure 3. Regions of the SCN and its outputs to the HPG axis. The SCN is divided into the core and shell region. In the core, VIP--expressing neurons project to the GnRH neurons, and in the shell, AVP-expressing neurons project to the AVPV Kiss1 neurons. The SCN projections to the GnRH and AVPV Kiss1 neurons help synchronize their clocks.

Strategic approach to target both the VIP and AVP neurons

Since both the VIP and AVP neurons in the SCN are thought to be involved in the HPG axis affecting reproduction, studying these two neuron populations together could help understand the relationship between the circadian and reproductive system. For this, we can target Neuromedin S (NMS) expressing neurons. NMS is a neuropeptide that is abundantly expressed in the SCN, and most SCN VIP and AVP neurons also express NMS (24). Moreover, the deletion of *Bmal1* in the NMS neurons leads to behavioral arrhythmicity, demonstrating that the NMS neurons have an important role in the timekeeping of the SCN (24). Therefore, NMS is a useful peptide marker to examine SCN control of reproduction, because (1) it is expressed in the VIP and AVP neurons which project to neurons involved in the HPG axis, and (2) the circadian gene *Bmal1* in the NMS neurons is critical in generating circadian rhythm.

How the circadian clock affects the reproductive system

Whole-body knockout of the core circadian gene, *Bmal1*, in mice results in arrhythmic estrous cycles and reduced ovarian weight, delayed pubertal onset, and infertility (25). The *Bmal1* knockout mice do not show an LH surge, and are infertile. However, the site of action of *Bmal1* is unknown. Our previous study showed that conditional knockout in Kiss1 and GnRH neurons had no disrupted reproductive health (26). Others have shown that loss of *Bmal1* in the pituitary also has no effect on reproduction (27). These findings led us to the hypothesis that the SCN projections to the HPG axis and the clock genes in the SCN are responsible for temporal regulation of reproduction, not endogenous clocks in peripheral populations. To study this, we chose to conditionally knock out *Bmal1* in the NMS neurons in female mice to examine the role of the SCN's main efferent neurons, VIP and AVP, in regulating the reproductive axis.

MATERIALS AND METHODS

Mice

All animal experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee. Bmal1$^{flox/flox}$ and NMS^{Cre} mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Bmal1$^{fl/fl}$-NMS^{Cre} mice were created by crossing NMS^{Cre} mouse and Bmal1$^{fl/fl}$ mouse. All mice placed with *ad libitum* access to food and water and were housed under LD 12:12 unless specified otherwise. All mice used for experiments were genotyped using PCR from their tail DNA. To evaluate for Bmal1$^{fl/fl}$ and NMS^{Cre} the following primers were used:

Bmal1 F1, 5' – CTGGAAGTAACTTTATCAAACTG – 3';

Bmal1 F2, 5' – CTCCTAACTTGGTTTTTGTCT – 3';

Bmal1 R, 5' – GACCAACTTGCTAACAATTA – 3';

NMS^{Cre} F, 5' – CCAAGTTAGCCTTCCATACACC – 3';

NMS^{Cre} R, 5' – AGACGGCAATATGGTGGAAAAT – 3';

NMS^{Cre} Control F, 5' – CCGCATCTTCTTGTGCAGT – 3';

NMS^{Cre} Control R, 5' – ATCACGTCCTCCATCATCC – 3'.

Bmal1$^{fl/fl}$-NMS^{Cre}-Rosa tdT mice were also created so that Rosa tdT is expressed in NMS^{Cre} neurons. To evaluate for Rosa tdT the following primers were used:

Control F, 5' – AAGGGAGCTGCAGTGGAGTA – 3';

Control R, 5' – CCGAAAATCTGTGGGAAGTC – 3';

Mutant F, 5' – CTGTTCCTGTACGGCTGG – 3';

Mutant R, 5' – GGCATTAAAGCAGCGTATCC – 3'.

Locomotor activity

Female Bmal1$^{fl/fl}$ and Bmal1$^{fl/fl}$-NMS^{Cre} mice were housed separately in individual cages; food and water were available *ad libitum*. Cages were placed in light-tight, ventilated chambers in constant darkness and the temperature was regulated at 22 ± 2℃. Cages were changed every 3-4 weeks. Locomotor activity was measured with passive infrared motion detectors installed in every cage for 60 days. At the end of the housing, these mice were euthanized to collect ovaries for histology. ClockLab (Actimetrics, Wilmette, IL) was used for circadian analysis. Free-running period was calculated by using a chi-squared periodogram to detect periods between 18 and 30 hours. Circadian amplitude was calculated using Fast Fourier Transform. Blackman-Harris filter and Power Spectrum were applied before normalizing the values to 1 (28).

Hormone assays

Mice in the diestrus stage were used to perform all the hormone assays. For the Kiss-10 challenge, the mice were weighed for dosing, and the basal LH was obtained through tail bleed. The mice were injected intraperitoneally with Kiss-10, the shortest biologically active form of kisspeptin, using a 2 mg/kg dose. Blood samples were obtained 10 minutes after the injection. The experiment was performed four to six hours after lights on (zeitgeber time (ZT) 4-6).

For the LH surge experiment, female mice were ovariectomized. Five days after the surgery, the mice were given 0.25 μg of β-estradiol in 100 μL of sesame oil and, on the following day, 1.5 μg of β-estradiol in 100 μL oil subcutaneously. On the next day, the mice were euthanized at ZT12 (lights off), and blood was collected to measure LH.

All the blood collected for the hormone assays was incubated for 20 minutes at room temperature to allow it to clot and then centrifuged at 2000 x *g* for 15 minutes. The serum

was collected in a separate tube and were placed at -20 ℃ until a multiplex assay was performed. LH concentration in the serum samples were measured using a Luminex Magpix on a Milliplex analyzer.

Fertility assay

Stages of the estrous cycle of the mice were measured by morning vaginal lavage for 24 consecutive days. The collected vaginal smears were dried and stained with methylene blue. They were analyzed for the presence of neutrophils, nucleated epithelial and anucleated epithelial cells according to Cora et al. (29). The presence of these cells was scored by two independent blinded observers for each day to observe for the cyclicity of the estrous cycle. Fertility was evaluated by mating the mutant female mouse with a wild type male mouse for 100 days. Days to first litter, the number of pups per litter, and total number of litters in 100 days were measured.

Ovarian histology

Mice in constant DD were euthanized and their ovaries were collected. These ovaries were immediately stored in a fixative solution (30% of 37% formaldehyde, 60% ethanol, 10% acetic acid). The following day, the ovaries were transferred to 70% ethanol and after 24 hours, were placed in fresh 70% ethanol solution. After the process of dehydration, the ovaries were embedded in a paraffin wax and were sliced at 20 μm using a microtome. They were placed on slides and dried overnight at 37℃. The ovary slices were stained with eosin-phyloxine and Harris hematoxylin solutions. After drying and cover-slipping with VectaMount Mounting Medium (Vector Laboratories, Burlingame, CA), the ovary slides were visualized and the number of follicles, Graafian follicles, and corpora lutea were

measured on every fifth section. The maximum number detected per feature was recorded for that animal.

Immunohistochemistry

Brains were collected in the early morning and were fixed in a 4% paraformaldehyde solution for overnight at 4℃. The next day, brains were transferred to a fresh 20% sucrose solution. After, the brains were washed in PBS and then embedded using OCT. The embedded brains were sliced to 40 µm using a cryostat. The collected brain slices were stored in a cryopreserving solution at -20℃ until the day of IHC. The day before beginning IHC, the stored slices were washed in PBS for overnight and on the first day of IHC, they went through the antigen retrieval process using Citra buffer (BioGenex, Fremont, CA). Afterwards, slices were incubated for 60 minutes in 5% goat serum (Vector Laboratories) for protein block. 1:1000 guinea pig anti-Bmal1 (Cat# AB2204, Millipore, Temecula, CA) was added to the slices and they were left for two days at 4℃. Goat anti-guinea pig (488), a fluorescent-conjugated secondary antibody, was added to the slices and incubated for 30 minutes. In between each step of the IHC, the slices were washed with PBS 3 to 4 times for 5 minutes and all the steps were carried out while light protected. After the final wash with PBS after the secondary, the brain slices were mounted on slides, dried and cover-slipped using Prolong solution with DAPI. During the whole process, the brains were kept light protected.

A Ti2 confocal microscope (Nikon Imaging Core, UCSD, San Diego, CA) was used to detect for the antibody fluorescence. Zeiss ZEN software was used to view and capture the images.

RESULTS

Bmal1$^{fl/fl}$-NMS^{Cre} mice have an arrhythmic behavioral circadian rhythm under constant darkness

To verify that the neuron-specific knockout of *Bmal1* in the NMS neurons abolishes circadian rhythm, we placed Bmal1$^{fl/fl}$ and Bmal1$^{fl/fl}$-NMS^{Cre} female mice under constant darkness in light-tight chambers (n =2 per group). Locomotor activity was recorded using a passive infrared motion detector for 60 days. Under DD, the control Bmal1$^{fl/fl}$ mice showed normal free-running period of around 23.5 hours (Figure 4A and 5A). However, the Bmal1$^{fl/fl}$-NMS^{Cre} mice displayed a disrupted free-running period (Figure 4B). The period with the highest amplitude was observed to 23.98 hours (Figure 5B), but their measured amplitude was around 4 times lower than the Bmal1$^{fl/fl}$ mice's amplitude. Further, the Bmal1$^{fl/fl}$-NMS^{Cre} mice also exhibited multiple significant peaks, instead of showing a consolidation of activity in a discrete period. Moreover, Bmal1$^{fl/fl}$ mice showed one activity cycle per day, but the arrhythmic Bmal1$^{fl/fl}$-NMS^{Cre} mice showed multiple activity cycles per day (Figure 6). This means that the Bmal1$^{fl/fl}$-NMS^{Cre} mice are actively moving multiple times in a day as a result of the locomotor rhythm amplitude being low. Overall, these findings confirm that deletion of *Bmal1* in the NMS neurons results in the disruption of the locomotor rhythm in female mice, presumably reflecting disruption of the SCN and potentially the efferent output.

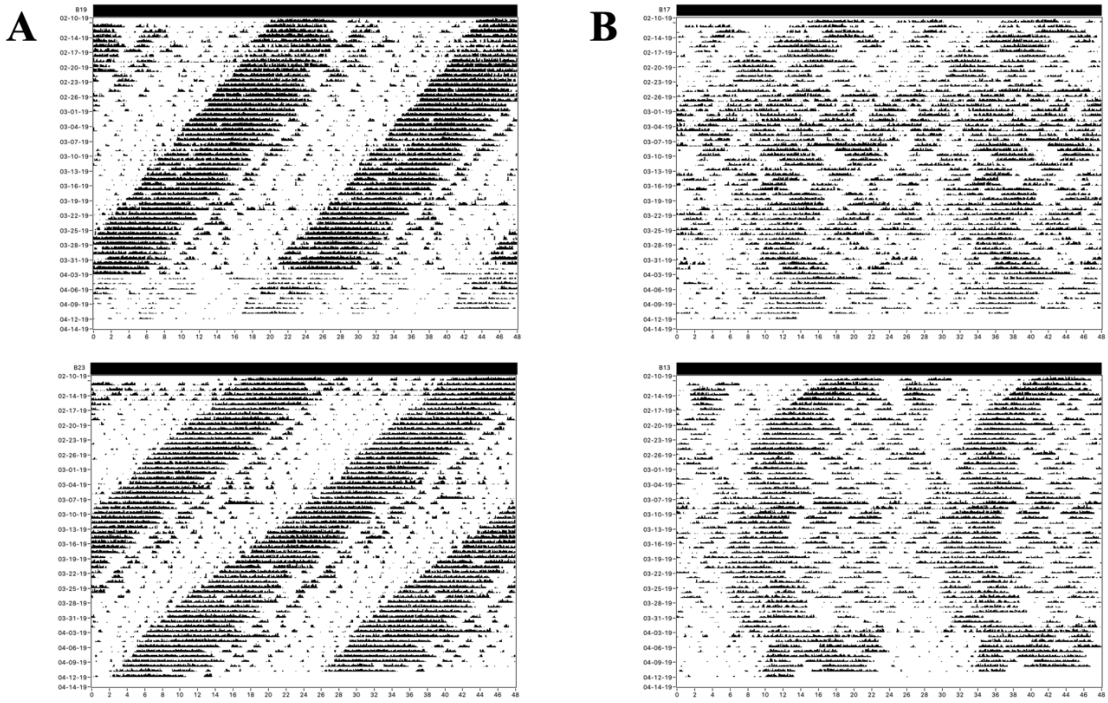

Figure 4. Bmal1$^{fl/fl}$-NMS^{Cre} female mice have disrupted locomotor circadian rhythms under constant darkness. Representative double plotted actograms of two separate (A) Bmal1$^{fl/fl}$ mice and (B) Bmal1$^{fl/fl}$-NMS^{Cre} mice. The mice were placed in a light-tight chambers with ad libitum access to food and water. Their locomotor activities were recorded using a passive infrared motion detector.

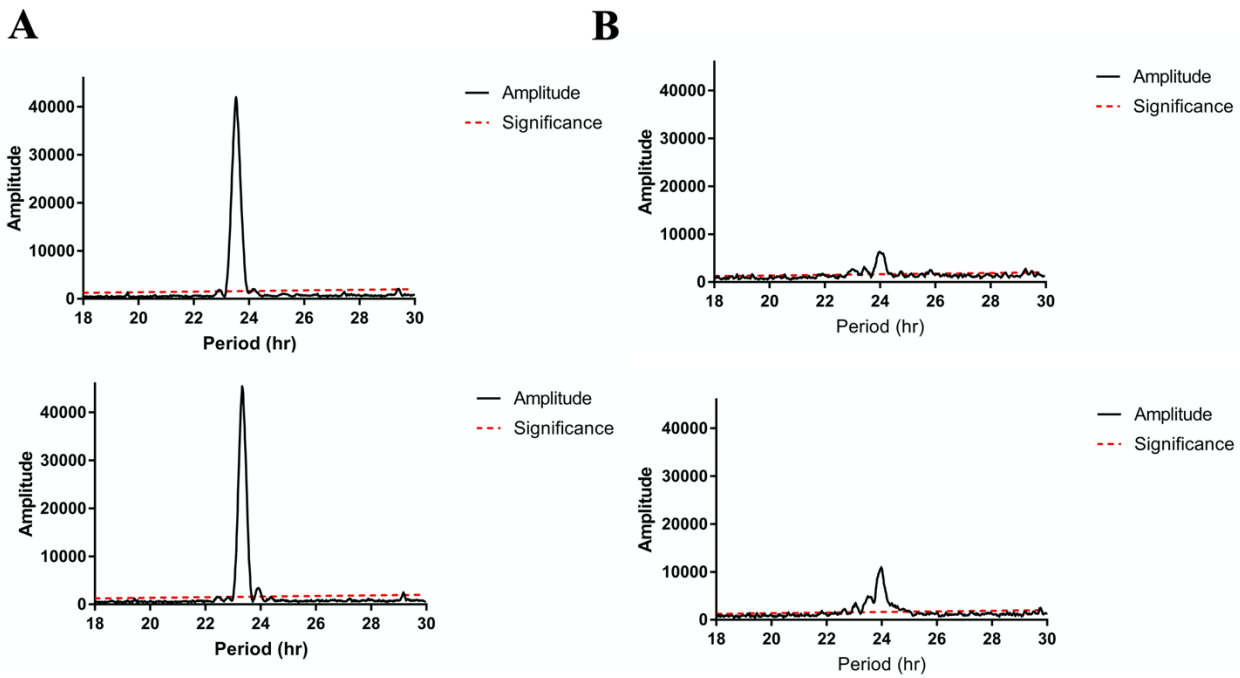

Figure 5. Bmal1$^{fl/fl}$-NMS^{Cre} female mice do not express a noticeable period under constant darkness. (A) Representative chi-squared periodograms of two separate Bmal1$^{fl/fl}$ mice showing a period of 23.53 (top) and 23.33 (bottom) hours with an amplitude of 41974 and 45351 respectively. (B) Representative periodograms of two separate Bmal1$^{fl/fl}$-NMS^{Cre} mice showing periods of 23.98. The line represents a threshold of 0.001 significance.

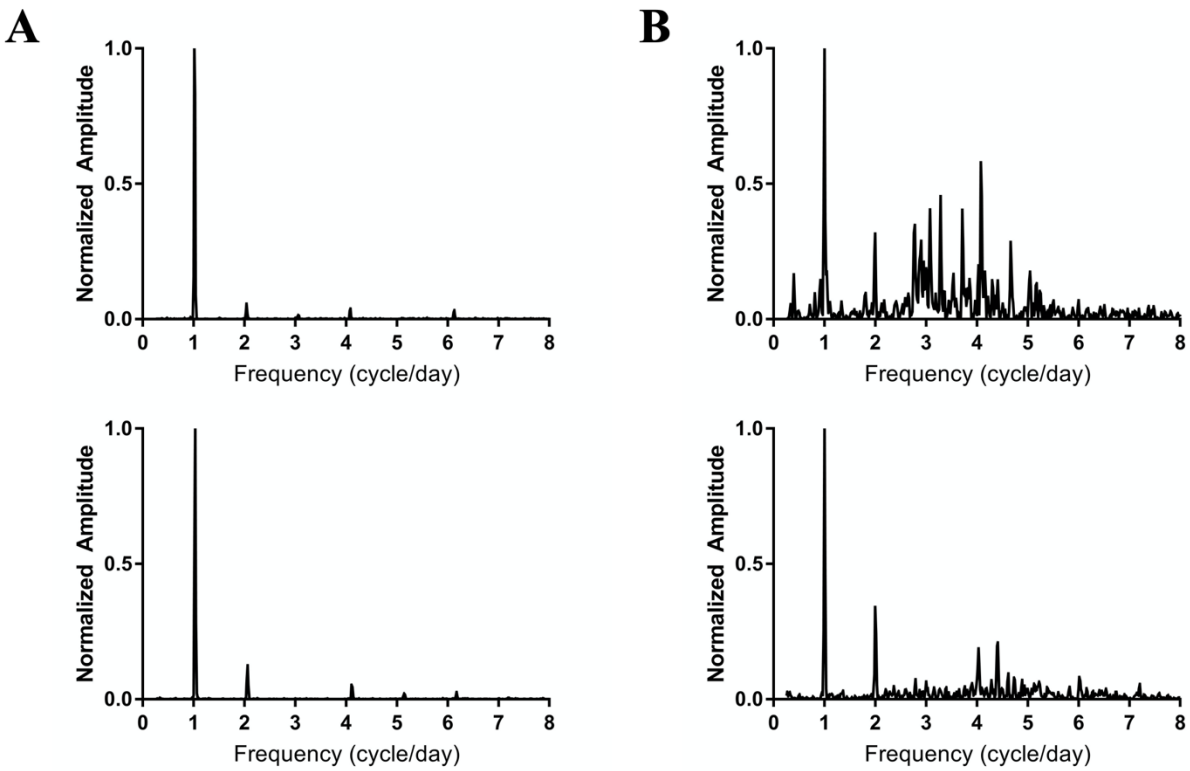

Figure 6. Bmal1$^{fl/fl}$-NMS^{Cre} female mice express weak and unprecise circadian amplitude. Representative circadian activity of two separate (A) Bmal1$^{fl/fl}$ mice and (B) Bmal1$^{fl/fl}$-NMS^{Cre} mice. It is possible to see one clear peak at one cycle per day in Bmal1$^{fl/fl}$ mice, but in Bmal1$^{fl/fl}$-NMS^{Cre} mice, many activity cycles can be observed in a day.

Bmal1$^{fl/fl}$-NMS^{Cre} mice are able to produce an LH surge and respond to kisspeptin

Having observed that the Bmal1$^{fl/fl}$-NMS^{Cre} mice have arrhythmic circadian rhythm in DD, we wanted to study how the HPG axis is affected in LD. The LH surge is important for ovulation and fertility and is not detected in whole-body *Bmal1* knockouts. To observe whether the Bmal1$^{fl/fl}$-NMS^{Cre} mice showed an LH surge, we performed an induced LH surge protocol on ovariectomized mice. Mice were ovariectomized to remove the endogenous estradiol so that the appropriate physiological concentration of exogenous estradiol can be given to elicit the positive feedback in the HPG axis, thus causing the LH surge. Bmal1$^{fl/fl}$-NMS^{Cre} mice showed a proper LH surge level at ZT12 (Figure 7A), as rodents have the surge in the evening time. Statistically, we found no significant difference in the LH level between the groups (Bmal1$^{fl/fl}$: 4.64 ± 2.29 ng/mL vs. NMS^{Cre}: 1.64 ± 0.23 ng/mL, p=0.24). In both groups, three of four animals were above the LH surge threshold of 1.5 ng/mL LH. Next, we tested the ability of the Bmal1$^{fl/fl}$-NMS^{Cre} mice to respond to kisspeptin during diestrus. The Bmal1$^{-/-}$ mice have increased responsiveness to kisspeptin, indicative of a disruption of the HPG axis. Blood was collected from Bmal1$^{fl/fl}$ and Bmal1$^{fl/fl}$-NMS^{Cre} mice before and 10 minutes after intraperitoneal Kiss10 injection (2 mg/kg) (Figure 7B). A two-way ANOVA revealed a significant effect of kisspeptin injection on LH levels, but there was no statistical differences among the groups (Bmal1$^{fl/fl}$: pre Kiss-10: 0.31 ± 0.15 ng/mL vs. post Kiss-10: 0.97 ± 0.36 ng/mL; NMS^{Cre}: pre Kiss-10: 0.22 ± 0.07 ng/mL vs. post Kiss-10: 0.80 ± 0.23 ng/mL). Overall, these suggest that the Kiss1 and GnRH neurons can be stimulated resulting in LH surge without the SCN's clock signal.

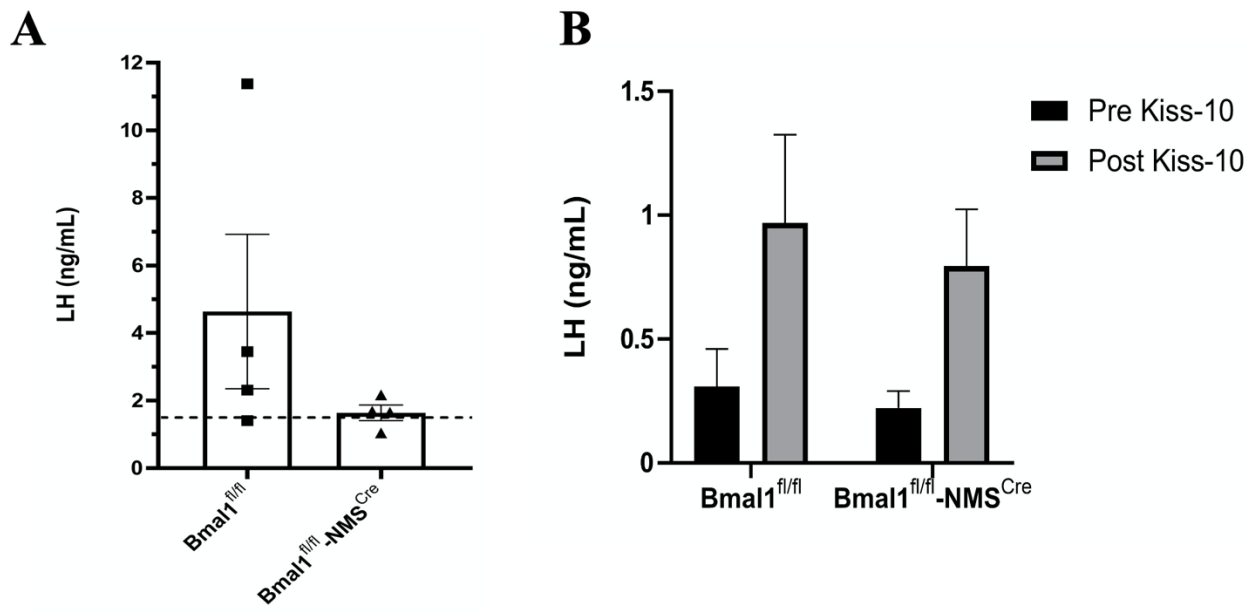

Figure 7. Bmal1$^{fl/fl}$-NMS^{Cre} females produce an induced LH surge and can be stimulated by Kiss-10. (A) LH levels measured on ovariectomized mice after estradiol injection to drive the LH surge. The mice were euthanized at evening (around ZT12) to collect blood and measure LH level (n=4, unpaired t-test not significant). Dashed line represents a LH surge threshold at 1.5 ng/mL. (B) LH levels measured before and after intraperitoneal injection of Kiss-10 at ZT8. Blood was collected by tail bleed to measure LH level (n=3 for Bmal1$^{fl/fl}$ and n=4 for Bmal1$^{fl/fl}$-NMS^{Cre}, two-way ANOVA significant for kiss treatment, $p < 0.05$).

Bmal1 NMS knockout mice have a rhythmic and undisrupted estrous cycle

To observe the circadian rhythm in reproduction, daily vaginal lavage was performed for 24 consecutive days on Bmal1$^{fl/fl}$-NMS^{Cre} mice to examine estrous cyclicity. The estrous cycle which sequentially consists of diestrus, proestrus, and estrus lasts about four to five days. Its stages are affected by levels of hormone involved in the HPG axis and the circadian system. Therefore, observing the estrous cycle allows examination of rhythms in reproductive activity. *Bmal1* knockout mice have prolonged estrus cycles and spend significantly more time in estrus than their wildtype counterparts (27). We found that the estrous cycle progressed normally in both Bmal1$^{fl/fl}$ and Bmal1$^{fl/fl}$-NMS^{Cre} mice (Figure 8A). We found no significant difference in the amount of time spent in each stage of the cycle between Bmal1$^{fl/fl}$ and Bmal1$^{fl/fl}$-NMS^{Cre} mice (Bmal1$^{fl/fl}$ diestrus: 46.88 ± 2.00 % vs. NMS^{Cre} diestrus: 31.67 ± 6.12 %; Bmal1$^{fl/fl}$ proestrus: 26.04 ± 3.56 % vs. NMS^{Cre} proestrus: 29.17 ± 3.23 %; Bmal1$^{fl/fl}$ estrus: 27.08 ± 3.61 % vs. NMS^{Cre} estrus: 39.17 ± 4.68 %) (Figure 8B). These results show that the progressive cyclicity of the female estrous cycle is not disrupted by the deletion of *Bmal1* in NMS neurons when analyzed in LD conditions.

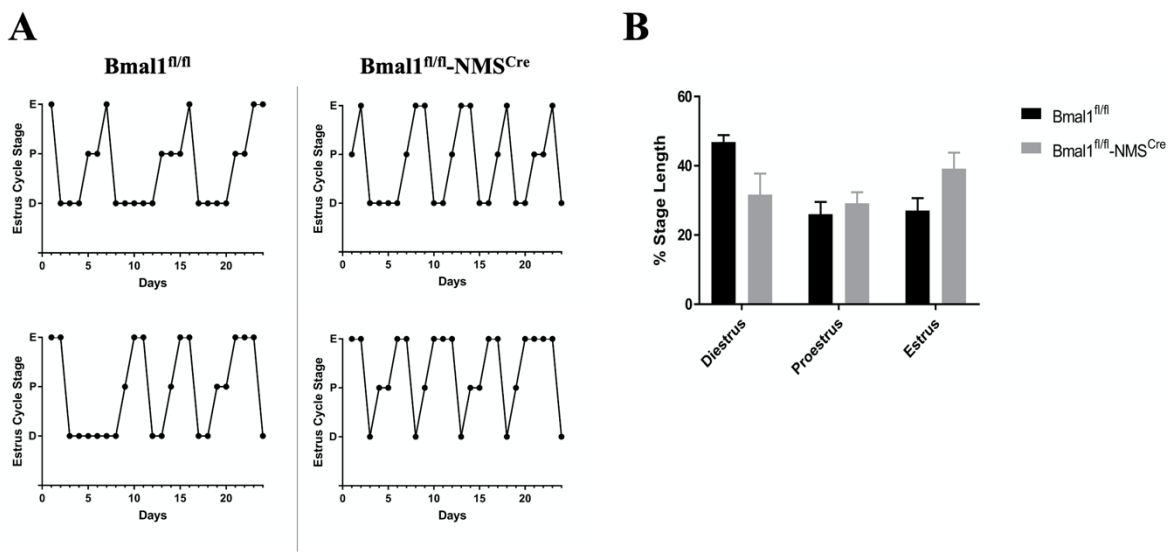

Figure 8. Bmal1$^{fl/fl}$-NMS^{Cre} mice have rhythmic estrous cycle in LD. (A) Representative observations of the estrous cycle in the *Bmal1* NMS KO mice. The stages were observed through 24 days of daily vaginal lavage that were stained with methylene blue. The presence of nucleated and anucleated epithelial cells and neutrophils was recorded to determine the stages (D: Diestrus, P: Proestrus, E: Estrus). (B) Average percentage length spent on each stage of the estrous cycle. (n=4 for Bmal1$^{fl/fl}$ and n=5 for Bmal1$^{fl/fl}$-NMS^{Cre}, two-way ANOVA not significant).

Conditional knockout of Bmal1 in the NMS neurons does not affect the fecundity of a female mouse

Despite having a normal estrous cycle, to investigate the reproductive success of the Bmal1$^{fl/fl}$-NMS^{Cre} females, we performed a fertility assay to examine their fecundity. When paired with a wild-type male mouse for 100 days, Bmal1$^{fl/fl}$ and NMS^{Cre} female mice did not show any significant differences in the time to produce their first litter (Bmal1$^{fl/fl}$: 31.38 ± 3.34 days, NMS^{Cre}: 28.83 ± 0.80 days, p=0.49) (Figure 9A), the number of pups per litter (Bmal1$^{fl/fl}$: 6.15 ± 0.45 pups, NMS^{Cre}: 7.10 ± 0.85 pups, p=0.36) (Figure 9B), or the number of litters produced during the experiment (Bmal1$^{fl/fl}$: 3.25 ± 0.48 litters, NMS^{Cre}: 3.50 ± 0.29 litters, p=0.67) (Figure 9C). These findings demonstrate that the female Bmal1$^{fl/fl}$-NMS^{Cre} mice have properly functional fecundity in LD conditions.

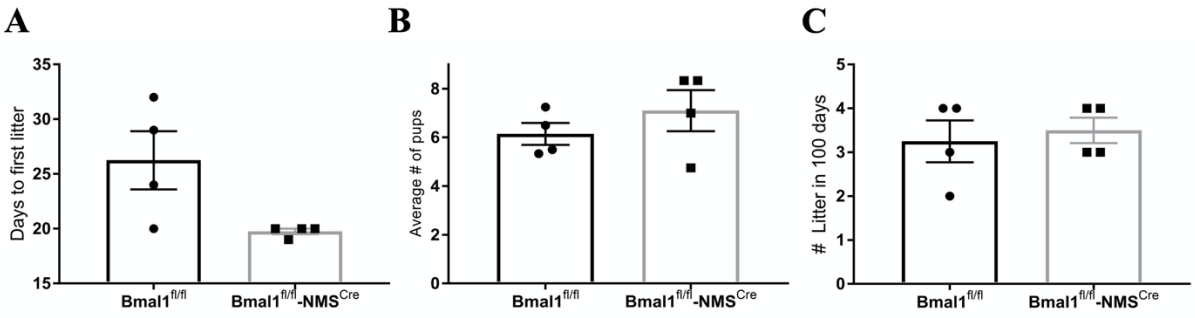

Figure 9. Bmal1$^{fl/fl}$-NMS^{Cre} mice have normal fecundity in LD conditions. (A) Number of days to first litter after pairing a female Bmal1$^{fl/fl}$ or Bmal1$^{fl/fl}$-NMS^{Cre} mouse with a wildtype male mouse (n=4, unpaired t-test not significant). (B) Number of pups produced per litter (n=4, unpaired t-test not significant). (C) Total number of litters produced in 100 days of pairing; litter produced 25 days after the male partner removal was recorded as well (n=4, unpaired t-test not significant).

Ovaries from Bmal1$^{fl/fl}$-NMS^{Cre} mice under constant darkness have fewer corpora lutea

Our Bmal1$^{fl/fl}$-NMS^{Cre} mice showed a proper induced LH surge and a fertile phenotype under a normal light/dark environment. However, because the mice showed arrhythmic locomotor behavior under constant darkness, we analyzed the ovaries in these mice for signs of ovulation to see whether they are still fecund in DD. As Graafian follicles release the ovum and become corpora lutea, we quantified the number of these in the ovaries (Figure 10). With unpaired t-test, we found no significant difference in the number of Graafian follicles and corpora lutea between Bmal1$^{fl/fl}$ and Bmal1$^{fl/fl}$-NMS^{Cre} ovaries (Graafian follicles: Bmal1$^{fl/fl}$: 3.00 ± 0.58 follicles vs. Bmal1$^{fl/fl}$-NMS^{Cre}: 3.80 ± 0.49 follicles, p=0.33; corpora lutea: Bmal1$^{fl/fl}$: 3.33 ± 0.95 corpora lutea vs. Bmal1$^{fl/fl}$-NMS^{Cre}: 1.20 ± 0.37 corpora lutea, p=0.09). Although not statistically significant at this number of animals, a lower number of corpora lutea is observed in Bmal1$^{fl/fl}$-NMS^{Cre} ovaries in constant darkness. This finding indicates that Bmal1$^{fl/fl}$-NMS^{Cre} mice may ovulate less than Bmal1$^{fl/fl}$ mice in constant darkness, and deserves future study.

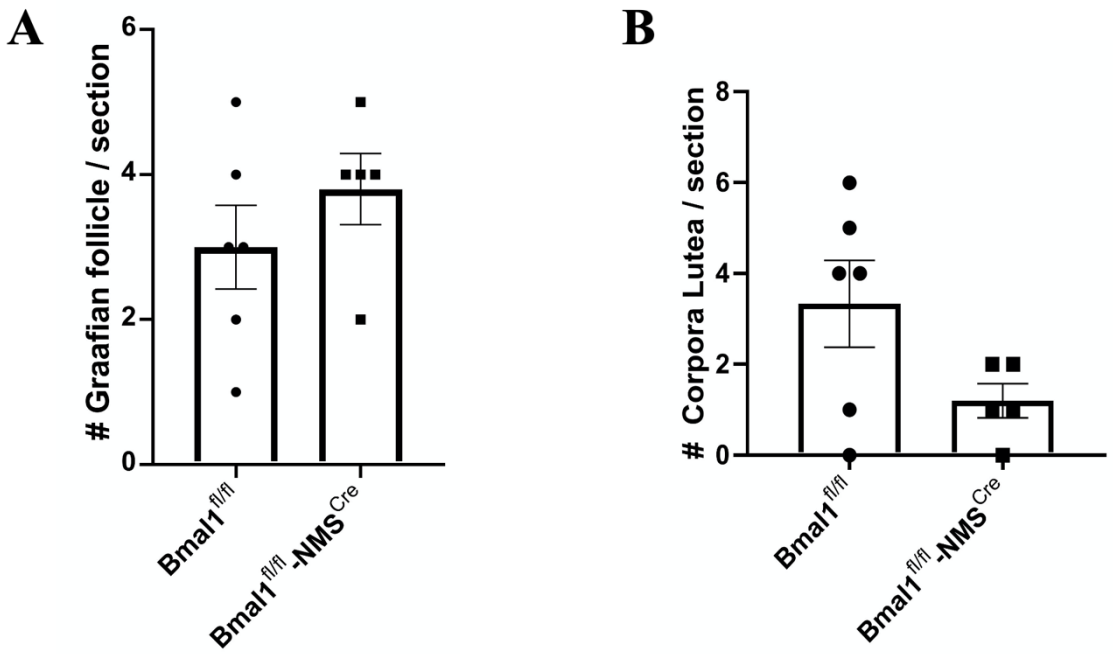

Figure 10. Ovarian histology of Bmal1$^{fl/fl}$-NMS^{Cre} mice under constant darkness. (A) Number of Graafian follicle per every fifth section of the ovaries in constant darkness (n=6 for Bmal1$^{fl/fl}$ and n=5 for Bmal1$^{fl/fl}$-NMS^{Cre}, unpaired t-test not significant). (B) Number of corpora lutea per every fifth section of the ovaries in constant darkness (n=6 for Bmal1$^{fl/fl}$ and n=5 for Bmal1$^{fl/fl}$-NMS^{Cre}, unpaired t-test not significant).

DISCUSSION

Deletion of *Bmal1* in the NMS neurons ablates the behavioral circadian rhythm in female mice

BMAL1 is a transcription factor that is critical for the molecular circadian clock and the resulting biological rhythms. Without BMAL1 in the SCN, the mammalian molecular clock is disrupted resulting in arrhythmicity. Lee (24) demonstrated that male mice with *Bmal1* deletion in the NMS neurons resulted in arrhythmic mice under constant darkness. We also demonstrated that female Bmal1$^{fl/fl}$-NMS^{Cre} mice have arrhythmic locomotor rhythms, as they expressed no precise period and weak amplitude under constant darkness (Figure 4, 5, 6). In addition, NMS is co-expressed in VIP and AVP neurons, which are located in the core and shell of the SCN, respectively. These neurons are important in synchronizing the clocks and maintaining circadian rhythm within the SCN and throughout the body. The VIP neurons are responsible for the SCN coupling with the shell's AVP neurons by acting on the VPAC2 receptors (10). We have not tested for the molecular rhythms in the SCN of the Bmal1$^{fl/fl}$-NMS^{Cre} mice, but our arrhythmic actograms (Figure 4) in the Bmal1$^{fl/fl}$-NMS^{Cre} mice suggest that the circadian rhythm in the SCN is disrupted. Moreover, the arrhythmicity due to *Bmal1* deletion would also indicate that the temporal inputs from the SCN to Kiss1 and GnRH neurons will be impaired. Hence, we have indirectly validated our Bmal1$^{fl/fl}$-NMS^{Cre} mouse model to have arrhythmic SCN expression and that is likely to create impaired temporal input to the reproductive neurons. However, we still need to validate our *Bmal1* knockout efficiency, because the NMS *Bmal1* knockout study by Lee (24) has shown 80% of *Bmal1* knockout in NMS neurons. The remaining NMS neurons with *Bmal1* could still compensate for the 80% knockout neurons providing SCN signals to few Kiss1 and GnRH neurons.

Single population deletion of *Bmal1* may be insufficient to affect fertility

There are many clock-controlled genes; thus, without an entrained circadian rhythm, expression of many genes would be altered. Kiss1 and GnRH neurons express BMAL1, and demonstrate cell-autonomous clock-dependent activity (19,30,31). VIP and AVP neurons send projections to GnRH and Kiss1 neurons, respectively. Whole body *Bmal1* KO mice do not show an endogenous or induced LH surge (26,27), but Bmal1$^{fl/fl}$-Kiss^{Cre} and Bmal1$^{fl/fl}$-GnRH^{Cre} mice do (26). We hypothesize that this is because *in vivo*, Kiss1 and GnRH neurons can still receive the circadian input from the master clock in the SCN. Therefore, we expected that without the SCN's temporal regulation, the Bmal1$^{fl/fl}$-NMS^{Cre} mice would fail to produce a LH surge at the appropriate time. However, the Bmal1$^{fl/fl}$-NMS^{Cre} mice showed an induced LH surge at the proper time (during lights off) and also increased LH levels after administering Kiss-10 (Figure 7). These data indicate that the HPG axis can act independently of the SCN's temporal regulation in response to exogenous stimuli. It is possible that the Kiss1 and GnRH neurons' endogenous clocks do not need to be entrained by the SCN to produce LH surge at the appropriate time. In addition, although there is no significant difference in LH surge between the groups, the lower LH level in the Bmal1$^{fl/fl}$-NMS^{Cre} mice can be due to a mistimed LH collection. Future studies will examine the timing of the LH surge in these mice in more detail, but it is important to note that this has no effect on fertility in these animals.

Bmal1$^{fl/fl}$-NMS^{Cre} female mice have normal fertility

Despite the loss of behavioral circadian rhythm, Bmal1$^{fl/fl}$-NMS^{Cre} females showed rhythmic estrous cycling through diestrus, proestrus, and estrus (Figure 8). This regular estrous cyclicity shows that the female reproduction is unaffected by *Bmal1* deletion in the NMS neurons in LD conditions. *Bmal1* or *Clock* knockout mice are observed to spend more

time in the estrus stage, demonstrating that clock proteins are involved in estrous cyclicity (27,32). However, the deletion of *Bmal1* in the NMS neurons affected the behavioral circadian rhythm, but it did not affect the reproductive circadian rhythm. Moreover, Bmal1$^{fl/fl}$-NMS^{Cre} females produced litters at the same rate as the control mice (Figure 9). Our female Bmal1$^{fl/fl}$-NMS^{Cre} mice bred successfully. Since the Bmal1$^{fl/fl}$-NMS^{Cre} mice could successfully produce LH surges (Figure 7) without interruption in the estrous cycle (Figure 8), we expected to find the mice fully fecund. Therefore, conditional knockout of *Bmal1* in these NMS SCN neurons does not cause infertility. It may be possible that the deletion of *Bmal1* in the NMS neurons does not affect fertility because, even though circadian signals are disrupted, Kiss1 and GnRH neurons have their own endogenous circadian clocks. Their clocks without the SCN's signals may be sufficient to keep the reproductive system functional. In addition, the disrupted behavioral rhythm occurred under constant darkness, but not under LD 12:12 cycle. Our experiments measuring reproductive parameters were performed under the LD conditions and this could explain how our Bmal1$^{fl/fl}$-NMS^{Cre} mice were fecund. Light may be synchronizing the SCN to allow circadian rhythms or the 20% remaining NMS neurons with *Bmal1* may entrain the clocks showing a rhythmic phenotype under LD.

Bmal1$^{fl/fl}$-NMS^{Cre} mice may have disrupted ovulation in constant darkness

We have performed the hormone assays, vaginal lavage, and fertility assays in a LD 12:12 cycle, and the Bmal1$^{fl/fl}$-NMS^{Cre} mice showed a fertile phenotype. Lee (24) showed that the male Bmal1$^{fl/fl}$-NMS^{Cre} mice were entrained by a LD 12:12 cycle. Because our the Bmal1$^{fl/fl}$-NMS^{Cre} mice also displayed normal locomotor rhythm in this condition (unpublished observation), we thought that light was allowing the clocks to be entrained. Hence, we have collected the ovaries to observe whether the mice in constant darkness that

had arrhythmic behavior ovulated normally (Figure 10). Although we found no statistically significant difference in the number of corpora lutea between groups, a trend to lower number of corpora lutea can be observed in ovaries from Bmal1$^{fl/fl}$-NMS^{Cre} mice placed in constant darkness. Furthermore, the analyzed ovaries were over-processed which made it harder to examine the histology. Nevertheless, Bmal1$^{fl/fl}$-NMS^{Cre} mice that showed arrhythmic locomotor behavior in constant darkness ovulated less than the rhythmic Bmal1$^{fl/fl}$ mice. Unlike the situation in LD 12:12 in which the Bmal1$^{fl/fl}$-NMS^{Cre} mice had normal reproductive phenotypes, these mice in constant darkness demonstrated reduced reproductive capacity. Therefore, this result potentially indicates that when the SCN clock is disrupted, female mouse reproduction is affected.

The thesis is coauthored with Mellon, Pamela and Tonsfeldt, Karen. The thesis author was the primary author of the thesis.

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