BIOLOGY OF THE BRAIN and MUSCLE ARNT-LIKE PROTEIN-1(BMAL1):

ROLES IN REPRODUCTIVE BIOLOGY

by

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ABSTRACT:

BIOLOGY OF THE BRAIN and MUSCLE ARNT-LIKE PROTEIN-1(BMAL1):

ROLES IN FERTILITY

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Brain and muscle Arnt-like protein (BMAL1) is a basic-helix-loop-helix (bHLH) transcription factor and member of the Per-ARNT-Sim (PAS) super family. *Bmal1* is an essential component of the circadian clock. Circadian rhythms play important roles in areas as diverse as cancer biology, reproductive biology, metabolism, energy balance and aging. Mice with global deletion of *Bmal1* display profound defects such as infertility, disrupted endocrinology, diabetes, early aging and arthropathy. In an effort to determine the roles of peripheral clocks and central clocks in reproductive biology, we used Cre/loxP recombination technology to genetically delete *Bmal1* specifically in various cell types with implicated roles in reproduction. In the first chapter of this thesis, we review what is currently known about the links among circadian rhythms, endocrinology and reproductive biology. In chapter two we show that the disruption of *Bmal1* in peripheral steroidogenic cells ($Bmal1^{fx/fx}Cre^{sf\text{-}1}$) results in female infertility due to implantation failure. $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females have lower progesterone levels than control females, and progesterone supplementation was able to rescue implantation. Transplantation of wild type ovaries into $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females results in 100% fertility, suggesting that ovarian *Bmal1* is the primary peripheral clock governing implantation and fertility in female mice. In chapter

three, we compare the relative roles of *Bmal1* in central nervous system ($Bmal1^{fx/fx}Cre^{nestin}$) and various peripheral cell types including the reproductive hormone axis ($Bmal1^{fx/fx}Cre^{sf\text{-}1}$), leydig cells or granulosa cells ($Bmal1^{fx/fx}Cre^{Amhr2}$), pituitary cells (somatotrophs, lactotrophs) ($Bmal1^{fx/fx}Cre^{rGhrhr}$) in male and female fertility. Only $Bmal1^{fx/fx}Cre^{nestin}$ males display defects in fertility (50% infertile). $Bmal1^{fx/fx}Cre^{sf\text{-}1,}$ $Bmal1^{fx/fx}Cre^{Amhr2}$, $Bmal1^{fx/fx}Cre^{rGhrhr}$ males are fertile, suggesting the importance of clocks in central nervous system for male fertility. $Bmal1^{fx/fx}Cre^{nestin}$, $Bmal1^{fx/fx}Cre^{Amhr2}$, $Bmal1^{fx/fx}Cre^{rGhrhr}$ females are fertile, consistent with the importance of ovarian *Bmal1* for female fertility. In chapter 4 we present future approaches to further elucidate the major themes of this thesis.

ABBREVIATIONS

Abbreviations used are: AVP = vasopressin, bHLH = basic-helix-loop-helix, CNS = central nervous system, DBP = D site albumin promoter binding protein, dpc = days post coitum, FSH = follicle stimulating hormone, GnRH = gonadotropin releasing hormone, hCG = human chorionic gonadotropin, HPG = hypothalamic-pituitary-gonadal, LH = luteinizing hormone, PMSG = pregnant mare serum gonadotropin, PRL = prolactin, SCN = suprachiasmatic nucleus, VMH = ventromedial hypothalamic nucleus, SD = standard deviation, SEM = standard error of mean, ZT = Zeitgeber time, SC = subcutaneous, + = wild type, ex = excised, fx = floxed.

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CHAPTER 1

Linking circadian clocks to fertility

INTRODUCTION

Organisms on earth have developed strategies to survive with optimal compatibility to the solar phases of light and dark. Among these strategies are innate circadian rhythms that control a variety of signal transduction processes so that an organism's biology is in phase with their illuminated world. These rhythms can typically be described by a repeated curve with a period of approximately 24 hours (Figure 1-1). Circadian rhythms influence almost every major important biological system, including those that control sleep-wake cycles, energy homeostasis, drug metabolism, aging, endocrinology and reproductive biology (1-6). Although difficult to prove, it seems likely that for a species to survive, its population is best served by maintenance of activity, feeding and reproduction rhythms that are not only in sync with their environment, but also with each other.

When we began these studies about five years ago, the original charge was to develop approaches to determine whether circadian biology had any impact on cancer outcome. In conceptualizing our approach we began to think about cancer models in the mouse where our expertise in genetics could have an impact. Specifically we focused on cancer models we had experience in, such as the dimethylbenzanthracene (DMBA) induced mammary cancer model, the diethylnitrosamine (DEN) induced hepatocarcinogenesis model and the *Apc*$^{Min/+}$ intestinal and colorectal tumor model (7-9). As we investigated these cancer model systems, early on, one thing became very clear. Each cancer model could be highly influenced by reproductive endocrinology (10-12).

We concluded that if we were ever to make sense of circadian influences on cancer, a preliminary understanding of the relationship between circadian biology and the reproductive system was our first task. We realized that the field of circadian biology and its role in

mammalian reproductive biology had never been thoroughly reviewed. This chapter was begun as an attempt to address this issue. As we learned more about this field, our interests began to evolve and the role of circadian clocks in reproductive success became of parallel importance. In particular, we became intrigued by the idea that an understanding of how peripheral clocks influence reproduction could ultimately have significant impact on fields as diverse as reproductive counseling, mammalian genetics, oncology and agriculture. Therefore, our review begins with a brief description of the workings of the mammalian molecular circadian clock. It then moves into a review of the experimental data documenting the potential importance of this circadian clock and reproductive biology. The review concludes with an attempt to understand which peripheral clocks may have most importance to reproductive biology. To this end, we review the evidence that the circadian clock may play a role in clocks within the reproductive axis (hypothalamus-pituitary-gonads), as well as those cellular clocks outside this axis that may also influence reproductive endocrinology.

FUNDAMENTALS OF CLOCK BIOLOGY

Physiological Outcomes of Circadian Rhythm Disruption.

There are emerging links between defects in circadian biology with negative health outcomes (13). Modern society and the use of electric lighting can alter an individual's circadian rhythms and activity/sleep cycles. Individuals with disturbed sleep, such as shift workers, show an increased incidence of diabetes, cardiovascular disease, breast cancer, prostate cancer and colorectal cancer (14-18).

Genome-wide association studies have found that genetic variants of the circadian rhythm regulatory pathway genes (details discussed in next section) contribute to a broad range

of human diseases. Human aryl hydrocarbon receptor nuclear translocator-like (*Bmal1, also known as Arntl or Mop3*) gene variants have been associated with bipolar disorder (19), diabetes, hypertension (20, 21) prostate cancer (22). Similarly, variants in the *Clock* gene have been associated with metabolic syndrome, obesity, diabetes, fatty liver, sleep disorder, breast cancer, and prostate cancer (23-25). Finally, *Per* gene variants are linked to obesity (26), sleep disorder, bipolar, postpartum bipolar disorder, breast cancer, and prostate cancer (22, 24, 27), while *Cry* gene variants are linked to breast cancer, and prostate cancer (22, 24).

Infertility affects approximately 1 in 10 couples (28, 29). Proposed causes of infertility include physical, environmental, and genetic factors, with both partners equally likely to be affected by these modifiers (30). As one potential contributing factors, female shift workers (such as nurses and flight attendants) have been reported to have a higher risk of irregular menstrual cycles and spontaneous abortions (31). Evidence that supports a role of circadian clocks in fertility include the observation that human *Bmal1* gene variants have been associated with a higher chance of miscarriages (32). Although there are no reports linking male infertility to circadian biology, shift work in men has been associated with higher prevalence of obesity and diabetes (33), which could predispose men to infertility issues. In men reporting symptoms of erectile dysfunction, 79% of the subjects are found to be overweight or obese (34). Furthermore, decreased androgen levels, reduced libido and erectile dysfunction are associated with male aging (35, 36). Male shift workers are reported to express “feeling” aging phenotypes earlier than workers on normal schedules (37, 38). It is possible that altered circadian rhythms could exert adverse effect on male fertility by inducing obesity and accelerating the aging process.

Molecular Basis For The Mammalian Circadian Clock System.

In mammals, circadian physiology is controlled by a molecular clock that is present in virtually every cell type. As a basic approach to keep all of these cellular clocks in synchrony, the molecular clocks of peripheral tissues are set by a master clock within the suprachiasmatic nucleus (SCN) of the hypothalamus (39). Evidence defining the SCN as the location of the master clock came from early neural transplantation experiments. In these pioneering studies, hamsters with SCN-lesions displayed arrhythmic locomotor activity, while retransplantation of a healthy SCN restored their circadian rhythmicity (39). The evidence that both master and peripheral cellular clocks can function in an autonomous manner came from the observation that SCN, liver, and lung explants and even single cells sustain rhythmicity when in isolation ("culture") for extended periods (40-45).

The last ten years has provided an explosion in our understanding of the mechanisms that underlie circadian rhythms. We now know that the molecular clock is running via a mechanism that is conserved in a wide variety of cell types. This molecular clock is dependent on multiple interlocking feedback loops (Figure 1-2). At the center of these feedback loops is a dimer of two basic-helix-loop-helix-PAS proteins known as CLOCK and BMAL1. This CLOCK:BMAL1 dimer transcriptionally activates feed-back components, such as those from the *Per* and *Cry* loci that inhibit that activity of the CLOCK:BMAL1 dimer (46), through E-box elements in their promoters (47, 48). Additional components on the negative limb include REV-ERB α/β and NFIL3. The REV-ERB family members repress transcription of *Bmal1* and *Nfil3* (49). NFIL3, represses transcription of ROR α/β (50). Additional components on the positive limb include *ROR α/β* and *DBP*. Transactivator ROR α/β drive rhythmic expression of *Bmal1* and *Nfil3*

transcription through binding to ROR response element (RRE) (51). Transactivator DBP drive expression of *Ror α/β* transcription through binding to D-box element (52).

These core clock components control transcriptional and translational feedback loops that guide the circadian expression of as much as 10-20% of the mammalian genome, regulating the oscillation of different transcripts in a cell type specific manner (53). These "physiological output" genes can be controlled through direct CLOCK:BMAL1 binding to regulatory E-boxes or DBP binding to D-box or *Ror α/β* binding to RRE elements that also oscillate as part of the core clock described above. "Physiological output" genes important in regulating circadian physiology include *vasopressin* (*Avp*), *steroidogenic acute regulatory protein* (*StAR*), *prolactin (Prl) and wee1.* BMAL1:CLOCK dimers can bind to E-box elements and drive gene expression of *Avp, StAR, Prl and Wee1* and thus regulate the rhythmic levels of AVP(54), glucocorticoid (55), prolactin (56) as well as the timing of cell division (57). Bioinfomatic analysis reveals the existence of E-box elements, RRE elements and D-box elements in regulatory sequences of many additional genes including *hydroxy-delta-5-steroid dehydrogenase 3 beta isomerase 1* (*Hsd3b1*), prolactin receptor (*Prlr*), insulin like growth factor (*Igf1*), *Leptin*, interleukin 5(*Il5*), *cytochrome P450 family 11 subfamily A polypeptide 1* (Cyp11a1) (58), implicating the underlying circadian control of a broad spectrum of biological processes.

Since the circadian clock has far reaching effects on the whole genome it is not a surprise that the disruption of circadian clock genes can produce profound phenotypes. In this regard, the global *Bmal1*$^{-/-}$ mice, in addition to loss of circadian locomotor rhythms, display a number of phenotypes including infertility, diabetes, increased sensitivity to chemotherapy and radiation, arthropathy, shorter lifespan, and early aging (59-63).

Redundancy of Molecular Clock Components

Inspection of the molecular clock (Figure 1-2) reveals that functional redundancy exists at almost every signaling step in the cycle. The extensive nature of redundancy may be a reflection of the fine tuning that is needed to keep a circadian clock in rhythm and may also be a reflection of the importance of this clock on the overall health of the organisms. The apparent singular exception to this redundancy is the *Bmal1* gene product. To date, *Bmal1* is the only gene for which functional redundancy does not exist. As a demonstration of this, the global *Bmal1*$^{-/-}$ mouse model for disruption of the molecular clock and its widespread use stems from the observation that it is the only genetic approach by which a single gene disruption leads to complete loss of locomotor rhythms (64). Interestingly, *Bmal1* has a close paralog, known as *Bmal2* or *Mop9* with similar dimerization and transcriptional properties as *Bmal1* (40, 65) . Expression of *Bmal2* in global *Bmal1*$^{-/-}$ (B1ko/B2Tg mice) can restore the circadian rhythmicity and several other phenotypes of *Bmal1*$^{-/-}$ mice (40). A current model to explain this observation is that the expression of *Bmal2* is E-box driven and requires BMAL1 for oscillatory expression. Thus the global disruption of *Bmal1* (*Bmal1*$^{-/-}$) also results in a functional disruption of *Bmal2*.

The other circadian component genes, such as *Per1* and *Per2*, *Cry1* and *Cry2*, *Clock* and *Npas2* require composite/simultaneous gene mutations to significantly disrupt locomotor rhythmicity in mouse models. For example, global knockout *Clock* mice (*Clock*$^{-/-}$) are still rhythmic in constant darkness. In contrast, mice lacking both *Clock* and *Npas2* (*Clock*$^{-/-}$; *Npas2*$^{-/-}$) display profound circadian phenotypes including irrhythmicity, male and female sterility, shorter life span, arthropathy, age-dependent weight loss, and early aging, similar to global *Bmal1*$^{-/-}$ mice (66). The lack of these phenotypes in mice lacking CLOCK alone suggests redundant contribution of NPAS2, presumably due to functional redundancy conferred by *Npas2*

(67). (66). Similarly, mouse models with single mutations of either *Cry1*$^{-/-}$ or *Cry2* $^{-/-}$ are still rhythmic in constant darkness while the single mutation of *Per1* or *Per2* results in varied rhythmicity (68). Yet again, double mutations of putatively redundant paralogs yields more significant rhythmicity defects. For example, *Per1/Per2* double-mutant mice (*Per1*$^{-/-}$*Per2*$^{-/-}$) and *Cry1/Cry2* double mutant mice (*Cry1*$^{-/-}$*Cry2*$^{-/-}$) are immediately arrhythmic (69). In summary, as an important survival strategy, it is not a surprise that there is functional redundancy between closely related genes in circadian physiology.

Although the circadian clock component genes are tightly interlocked and could be substituted by their paralogs, the disruption of different components do display gene specific phenotypes. For example, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double mutants and the *Per1*$^{-/-}$*Per2*$^{-/-}$ double mutants, although arrhythmic, don't display the arthropathy observed in the global *Bmal1*$^{-/-}$ or the *Clock*$^{-/-}$ *Npas2*$^{-/-}$ double mutants.

The functional consequences of substitution of circadian clock genes also display tissue-specific effects. For example, expression of *Bmal2* in *Bmal1*$^{-/-}$ mice (B1ko/B2Tg mice) can rescue locomotor and metabolic rhythmicity, but not the rhythmicity in SCN or liver explants (40). In addition, in *Clock*$^{-/-}$ mice, while gene expression displayed by Per2:luciferase is still rhythmic in SCN the rhythms don't exist in lung or liver explants (44, 70, 71). This means that the functional paralog of *Clock, Npas2* can substitute *Clock*'s function in the SCN, but not in liver or lung, suggesting there are differences between the clock machinery of different tissues.

Modification of the Circadian Clock by Other Signaling Pathways

The circadian clock system interacts with other biological systems such as food intake, hormone cycles, etc. For example, glucocorticoids (an adrenal hormone) can restore rhythmicity

in liver explants in *Bmal2* rescued *Bmal1*$^{-/-}$ mice (B1ko/B2Tg) mice. Moreover, disruption of the adrenal clock by down regulating *Bmal1* in adrenal specifically results in arrhythmic glucocorticoid release (55), indicating the crosstalk between the liver clock and adrenal hormone system (72). LH can induce the rhythmic gene expression of *Per1* and *Bmal1* in chicken ovarian granulosa cell culture (73). These two observations suggest that systemic hormone levels can regulate tissue-intrinsic clocks.

Another example is that restricted feeding not only alters circadian behavior but also extends life span (74). Finally, testosterone and estrogen can alter circadian behavior (75). Androgen, estrogen, progesterone receptors have been found in the SCN (76). In summary, specific circadian component genes might respond to specific signals in specific tissues to integrate these signals into the functioning of the whole organism.

Mutations In Molecular Clock Genes Affect Reproductive Outcomes.

It makes sense to think that circadian biology and reproductive biology would be intimately linked. To support this idea, we offer the obvious point that for successful mating to occur, members of the same species must interact, a process which is enhanced by synchrony of activity phases within a community. In support of this concept is the observation that many rodent models harboring genetic mutations of circadian core components display adverse reproductive phenotypes. For example, both male and female global *Bmal1*$^{-/-}$ and *Clock*$^{-/-}$*Npas2*$^{-/-}$ double mutant mice are infertile (70). In addition to displaying implantation failure, *Bmal1*$^{-/-}$ females display various reproductive defects, including delayed vaginal opening, a greater frequency of disrupted estrus cycles, reduced ovarian weights, and reduction in both mammary ductal length and number of branches (58). Moreover, ~20% of *Bmal1*$^{-/-}$ females displayed

continuous diestrus or absence of cornification (>5 days), indicating non-cycling estrous cycles (58). These non-cycling female mice display mammary glands with significantly fewer branches and ductal length and significantly bigger fat pads. Their uteri were significantly thinner than wild type controls and other cycling *Bmal1*$^{-/-}$ females. In addition, global *Bmal1*$^{-/-}$ males display hypogonadism, low testosterone, FSH, sperm count, and high LH (77). Furthermore, global *Bmal1*$^{-/-}$ males have low libido evidenced by observation of fewer copulation plugs in wild type female partners. In summary, the disruption of circadian rhythms result in defects in various aspects of reproductive biology.

Additional evidence that the molecular clock plays a role in rodent fertility comes from other mouse models of disrupted circadian rhythm. In this regard, a dominant negative CLOCK protein variant devoid of the exon 19, *Clock*$^{\Delta 19}$, renders CLOCK-BMAL1 heterodimers functionally defective (78). This dominant negative mutant *Clock*$^{\Delta 19/\Delta 19}$ mutation confers obesity, disrupted estrous cycles, elevated rates of fetal reabsorption and pregnancy failure on female mice (79). Similarly, as *Per1*$^{-/-}$ and *Per2*$^{-/-}$ female mice age, they display smaller litter sizes as compared to their wild type controls (80). The decline in reproductive capacity with increasing age in these mice has been attributed to an accelerated failure of luteal support of the uterus (81). These observations mean that in addition to *Bmal1*, the disruption of other circadian component genes also results in defects in reproductive biology.

CIRCADIAN CONTROL OF THE REPRODUCTIVE AXIS.

Mammalian reproduction requires complex, reciprocal interactions among gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus, the anterior pituitary, and the testes or ovaries. The interplay of these cell types is collectively referred to as the reproductive axis. In the

classical view of reproductive axis, GnRH released from hypothalamus GnRH neurons binds to GnRH receptor (GnRHR) on the pituitary gonadotropes and induces the production of gonadotropins including luteinizing hormone (LH) and follicle stimulating hormone (FSH) (82). In turn, LH and FSH bind to LH receptors and FSH receptors in the gonads (ovaries or testis) and induce production of sex hormones such as estrogen, progesterone and androgens (83, 84). Sex hormones can subtly inhibit or increase the secretion of GnRH or gonadotropins depending on developmental stages of the gonads (85) (46), forming classical feedback loops.

Given the evidence that circadian clocks can regulate reproductive biology, it is important to review the potential steps along the reproductive axis where such an interaction might occur. These potential links between circadian clocks and the reproductive axis are shown in figure 1-4,5 and are described below.

The Hypothalamus

Gonadotropin-releasing hormone. In mice GnRH neurons are a small and diffuse population of GnRH-expressing cells scattered over a large area of the medial hypothalamus and basal forebrain, with a dense population in the preoptic area (POA) (86) (figure 1-6,7). The evidence that circadian clocks may regulate the function of GnRH neurons includes the following observations. First, *Bmal1* and *Per2* oscillate with a 24-hour period in the GnRH population, in vivo and in vitro (87). Second, GnRH release in cocultures of the POA and SCN exhibits a significant circadian rhythm in phase with *Avp* expression (88), an output gene of circadian clocks (89). Further, the GT1-7 cell line, which is an in vitro model of GnRH neurons displays decreased GnRH pulse frequency after expression of $Clock^{\Delta 19}$. Overexpression of *Cry1* in GT1-7 cells increased GnRH amplitude. Finally functional E-box elements exist in promoters

of the *Gnrh* and thyroid transcription factor 1 (TTF1) genes (90). The TTF1 protein, a transactivator of *Gnrh* transcripts, is a component of the molecular machinery controlling circadian oscillations in *Gnrh* gene transcription (90). The abundance of *Ttf1* mRNA varies in a diurnal fashion in the POA of the rat in vivo. BMAL1, CLOCK and PER1 repress *Ttf1* promoter activity while CRY1 activate *Ttf1* transcription. In turn, TTF1 represses transcription of *Rev-erbα* (91). These observations suggest that the expression of *GnRH* is under circadian control.

Comparing the reproductive phenotypes of *Gnrh* mutant (hpg mice) and the molecular clock mutants could add to our understanding of the relationship between GnRH and circadian clocks (Table 1-1). Hypogonadal (hpg) mice have a truncation of the *Gnrh* gene, and undetectable LH and FSH secretion (82, 92). In hpg males, testes fail to undergo normal postnatal development (arrested spermatogenesis) and display 98% and 99% decreases in testis and seminal vesicle weight, respectively. Transplanting normal POA tissues containing GnRH neurons into hpg males (hereafter hpg/POA mice) can induce gonadotropin levels, gonadal growth, and steroidogenesis, but fail to induce masculine sexual behavior. Testosterone supplementation of adult hpg/POA males does not restore fertility, however, the infertility of hpg males can be rescued if neonatal testosterone treatment is added (93), suggesting the importance of neonatal androgenization for male fertility. More specifically, perinatal androgens and their estrogen metabolites direct the developmental trajectory of sexually dimorphic neural substrates and masculinize the male neural circuitry and behaviors.

In contrast, male mouse mutants carrying mutations of *Per, Cry, Clock* mutations display only moderately reduced fertility or even normal fertility. The mutants with most severe male reproductive phenotype are the global *Bmal1*$^{-/-}$ mice and *Clock*$^{-/-}$*Npas2*$^{-/-}$ double mutants. The global *Bmal1*$^{-/-}$ males, although able to occasionally sire a litter, are infertile in 99% of the

breeding experiments (66, 77). However, global *Bmal1*$^{-/-}$ males have functional sperm, bigger testis and seminal vesicle (~21% and ~42% decreases respectively), suggesting modest but suboptimal spermatogenesis and gonadal development. It is possible that the difference between hpg males and molecular clock mutant males could be due to incomplete overlapping of *Bmal1* and *Gnrh* expressions or functions. "Leaky" expression of GnRH in cells that don't express *Bmal1* can support moderate although not optimal testis development. However, the sexual behavior of *Bmal1*$^{-/-}$ is similar to the hpg/POA males, evidenced by lack of copulation plugs in their female partners, suggesting that the leakage of GnRH in neonatal stage could not support normal neonatal brain development. It will be interesting to see to whether neonatal or perinatal testosterone injection can rescue the infertility of *Bmal1*$^{-/-}$ males.

In contrast to hpg males, hpg female mice with POA grafts (hpg/POA females) could mate, ovulate and bear litters (94). Interestingly, LH levels of hpg/POA females are lower than that needed to induce spontaneous ovulation. Instead, ovulation is driven by a mating-induced reflex release of hormone, a response that appears to be controlled by neural circuitry distinct from a classical LH surge. Similarly, *Clock*$^{\Delta 19/\Delta 19}$ female mice fail to display a spontaneous LH surge but can successfully ovulate. In our unpublished data, conditional central nervous system *Bmal1* knock out females (*Bmal1*$^{fx/fx}$*Cre*^{Nestin} females) are also fertile, suggesting that, although the spontaneous LH surge might be affected by disruption of circadian clock, the female mice can compensate either by residual GnRH, LH or reflex release of hormones. For example, olfactory environment and coitus can trigger ovulation and luteinization (95). In summary, comparison of the reproductive phenotypes of those different female models could help us understand the functions of GnRH in control of in the ovulation process.

Kisspeptin. Kisspeptins are a family of neuropeptides that are mainly expressed in discrete neuronal populations of the hypothalamus, such as POA, paraventricular nucleus and arcuate nucleus (96, 97). GPR54 is a G-protein coupled receptor for Kisspeptin. The importance of Kisspeptin–GPR54 signaling for reproductive biology comes from the observations that mutations of *Gpr54* in humans cause hypogonadism. In addition, global *Kiss*$^{-/-}$ and *Gpr54*$^{-/-}$ mice are infertile (98). Kisspeptins and GPR54 can regulate GnRH release and are necessary for proper male-like development of sexually dimorphic traits during perinatal development (97, 99).

The following studies establish a potential basis for a linkage between circadian clocks and kisspeptin-GPR54 signaling. First, kisspeptin neurons in the POA exhibit estrogen-dependent circadian patterns of cellular activity and the patterns coincide with the LH surge (100). Second, the SCN targets kisspeptin neurons via vasopressinergic (i.e., AVP) projections (100). Finally, the GnRH system exhibits time-dependent sensitivity to kisspeptin stimulation. In summary, kisspeptin signalling system is under circadian control.

Comparing the reproductive phenotype of global *Bmal1*$^{-/-}$, hpg and global *Kiss1*$^{-/-}$ mice reveals that *Bmal1*$^{-/-}$ mice share greater similarity with *Kiss1*$^{-/-}$ mice than with hpg mice (101) (table 1-1). Like *Bmal1*$^{-/-}$ males, *Kiss1*$^{-/-}$ males display normal gonadal histology. In females, *Kiss1*$^{-/-}$ mice, like *Bmal1*$^{-/-}$ females, display phenotypic heterogeneity. Half of *Kiss1*$^{-/-}$ females have markedly reduced gonadal weights, similarly to those of non-cycling *Bmal1*$^{-/-}$ female mice. The remaining *Kiss1*$^{-/-}$ females exhibit persistent vaginal cornification (indicative of poor luteal ovarian function) but have gonadal weights comparable to wild-type females.

One interesting study on kisspeptins reveals that a low level (10%) of GnRH neurons is enough for female reproductive development (102). By expressing diphtheria toxin A (DTA, a

toxin whose expression will kill cells) under the direction of the kisspeptin promoter, a kisspeptin neuron knockout mouse model was generated (hereafter Kiss/DTA mice). Kiss/DTA females displayed significantly smaller ovaries and a decreased number of GnRH neurons (~10%) compared to littermate controls, consistent with previous studies that kisspeptins regulate the functioning of GnRH neurons and thus regulate puberty onset and fertility. However, the timing of puberty onset and fertility is normal in Kiss/DTA females, suggesting that ~10% of the GnRH neuron repertoire is sufficient for reproductive development. In contrast, acute kisspeptin neuron ablation at postnatal 20 days of age or later resulted in acyclicity and infertility, suggesting that a developmental compensation for kisspeptin neurons in Kiss/DTA females occurs before P20. This study adds to our understanding about the complicated compensation and redundancy in reproductive biology and suggests that fine mapping of the functioning of circadian clocks, GnRH neurons and Kisspeptin neurons in embryonic development stage is required.

The Pituitary.

In the classical reproductive axis, the pituitary receives signals from the hypothalamus and relays these signals to gonads. Many anterior pituitary hormones in humans are released episodically over the 24 hour period, with the frequency and magnitude of the episode controlled in part by circadian rhythms and sleep-onset cycles in human. Examples of such relays include the release of adrenocorticotrophic hormone (ACTH), growth hormone (GH), prolactin, and gonadotropins (103-106).

The GnRH receptor (GnRHR). The GnRHR, a member of G-protein coupled receptor family, is expressed on the surface of pituitary gonadotrophs. Following binding of GnRH, GnRHR associates with G-protein and ultimately activates LH and FSH release. Evidence that

circadian clocks can regulate GnRHR include the observation that E-box elements are found in the promoter of the GnRHR gene (107, 108). Moreover, transient overexpression of the dominant-negative, *Clock*$^{\Delta 19}$, or the *Per1* markedly reduces GnRHR expression in a dose-dependent fashion, implicating direct control of expression of GnRHR (108).

Gonadotropins. The gonadotropins (LH and FSH) are produced in pituitary gonadotropes. In humans, diurnal rhythms of LH and FSH secretion are apparent before the onset of puberty (106, 109). In juveniles and adult male mice, FSH exhibits significant diurnal variation. These rhythms could come from the rhythmicity of GnRHR, however, in adult male mice, these diurnal patterns are less obvious. Studies show that other factors (such as female urine stimulation) can induce the LH surges in adult male mice (110, 111). It is well established that the circadian clocks control the proestrus LH surge and through this surge, ovulation in laboratory animals (73, 112). Support for this idea comes from the observation that in mice and rats, the LH and FSH surge occurs only in the afternoon of the proestrous day (113) (Figure 1-4). Moreover, *Clock*$^{\Delta 19/\Delta 19}$ females lack the LH surge, yet, injection of AVP in the afternoon of the proestrous day can rescue an LH surge in *Clock*$^{\Delta 19/\Delta 19}$ females (114). This observation suggests that that AVP can act as a circadian signal to regulate the proestrous release of LH. It has been proposed that that the precise timing of LH surge is mediated by SCN neuronal projections to GnRH neurons (115).

It is worthwhile noting that, although it is established that ovulation is under circadian regulation, female mice harboring mutations in core clock genes such as *Clock*$^{\Delta 19/\Delta 19}$, *Bmal1*$^{-/-}$, *Per1*$^{-/-}$, *Per2*$^{-/-}$, *Cry*1$^{-/-}$, *Cry2*$^{-/-}$ do ovulate, suggesting that non-classical compensating pathways exist for ovulation.

Prolactin (PRL) And Growth hormone (GH). PRL and GH are secreted by pituitary lactotrophs and somatotrophs, respectively. The importance of PRL for female reproduction comes from the observations that the global *Prl*$^{-/-}$ and *Prlr*$^{-/-}$ females are infertile due to implantation failure. Furthermore, they also display irregular estrous cycles, impaired mammary gland development, reduced fertilization rate of oocytes, and defective preimplantation embryonic development (116). In contrast to females, *Prl*$^{-/-}$ males are fertile. Although *Prlr*$^{-/-}$ males display delayed fertility but are ultimately fertile (117).

Evidence for the importance of growth hormone (GH) in reproductive biology comes first from the observations of "Laron syndrome". Laron syndrome carrying mutations that inactivate the growth hormone receptor is associated with low gonadotropin levels and small gonads (118). Treatment of Laron syndrome with the GH downstream factor, insulin-like growth factor (IGF-1) can increase gonadotropin secretion, gonad size and gonadal hormone (118). It is said that the GH/IGF-1 axis is a major signaling pathway that connects somatic development to the GnRH-releasing system during sexual maturation. Second, GH-Receptor-KO mice can reproduce, but their breeding performance is reduced, particularly in females. GH resistance leads to quantitative deficits in reproductive development and functions, but does not preclude fertility in either sex. Finally, reductions in GH secretion are associated with high rates of delayed onset of menarche, oligo-ovulation, and infertility in women (119, 120).

Additional evidence for the importance of PRL and GH comes from the Ames (df/df) and Snell dwarf (dw/dw) rodent models. In Df/df and dw/dw mice, carrying mutations in transcription factors Prop-1 and Pit-1 respectively, lack prolactin, growth hormone and thyroid stimulating hormone (TSH). Injection of df/df and dw/dw females with PRL rescues infertility and lactation deficiency in both strains (121). In addition, GH or IGF-1 administration has been

shown to increase the concentration and motility of spermatozoa in epididymis in dwarf mutants. IGF-1 is also capable of upregulating steroidogenesis by stimulating LH receptor expression in pig Leydig cells in vitro and by enhancing the activity of steroidogenesis enzymes. It is worth noting that PRL and GH are likely to have overlapping roles in providing regulatory inputs to the hypothalamic-pituitary-gonadal axis (122).

The evidence that circadian clocks regulate GH secretion comes from the observation that human serum GH levels display 24-hour profiles with elevated levels during sleep (103). The evidence that circadian clocks control PRL secretion comes from a number of observations. Rhythmic expressions of *Per2* was found in neuroendocrine dopaminergic neurons, which control prolactin secretion (123). Human serum PRL displays 24-hour profiles with elevated levels during sleep (103, 104). Virgin female mice display a PRL surge only in the afternoon of proestrous day (113). During pregnancy PRL follows daily biphasic surges. In addition, SCN lesion blocks the PRL surge (124). Furthermore, functional E-box element exist in the promoters of *Prl* genes (56). Finally, $Clock^{\Delta 19/\Delta 19}$ dams secrete less milk (5). The nursing behavior is also defective in $Clock^{\Delta 19/\Delta 19}$ dams, which might be a result of defective PRL levels.

The Testis.

Ninety five percent of serum testosterone is synthesized by the Leydig cells in the testis. The remainder is produced by adrenal cortex. Androgen production by adult Leydig cells is regulated by GnRH from the hypothalamus and LH from the pituitary. As mentioned above, pulsaltile GnRH is secreted into the hypophysial portal system. Accordingly, LH secrection is also pulsatile. Robust episodes of LH release precede testosterone peaks. Both the frequency and amplitude of the pulses are influenced by steroids (testosterone and estrogen), forming positive or negative feedback loops (figure1-4). There is a trend of lower levels of testosterone with

aging, probably due to fewer LH discharges which might result from the slowing of GnRH pulse generator (125). Circadian clocks, known to regulate aging process, could thus regulate testosterone levels, but the detailed mechanism is still lacking.

Whether testosterone levels follows a circadian rhythm is dependent on species and developmental stage. In rhesus macaques, testosterone secretion follows a diurnal pattern, but this pattern is not obvious in adult male mice, rats and ferrets (109, 125-127). The expression of *Per1,Per2*, *Bmal1*, *Clock* and *Cry1* in mouse testis is not rhythmic (128, 129). However there is a report that BMAL1 protein levels display daily rhythms in Leydig cells in the testis (77). Further expression study on specific cell type will be informative on whether cells in testis follow circadian rhythms of functioning.

The best evidence that circadian clocks may directly control testis function comes from the observation that testis *StAR* expression is reduced in global *Bmal1*$^{-/-}$ male (77). The product of the *StAR* gene is a major regulator of steroidogenesis as it is a cholesterol translocator in mitochondria. Evidence that *StAR* is regulated by circadian rhythms is supported by the observation that the CLOCK:BMAL1 dimer can bind to E-box elements of its promoter in vitro (55). Functional relevance of this interaction is supported by the observation that global *Bmal1*$^{-/-}$ males display lower testosterone levels (77). *StAR* is also expressed within steroidogenic glia and neurons in the brain, implicating star in the production of neurosteroids and possibly playing a role in the regulation of libido.

It is interesting to compare the phenotype of global *StAR*$^{-/-}$ and *Bmal1*$^{-/-}$ mice (table 1-1). Similar to *Bmal1*$^{-/-}$ mice, *StAR*$^{-/-}$ males display normal testis, epididymis, vas deferens and mature spermatids within the epididymis, indicating that *StAR*$^{-/-}$ testes retain sufficient androgen

biosynthesis to support germ cell maturation. However, the male-to-female sex reversal of external genitalia in *StAR*$^{-/-}$ males indicates impaired androgen production in utero. The differences in virilization between *StAR*$^{-/-}$ and *Bmal1*$^{-/-}$ males suggests that, although BMAL1:CLOCK dimer directly regulate *StAR* expression, other mechanisms for the transactivation of *StAR* exist in the absence of a functional *Bmal1* and this compensation is enough to fully virilize male external genitalia.

The Ovaries

In the reproductive axis, the ovaries receive signals via pituitary LH and FSH and in turn secrete sex steroids (such as estrogen and progesterone). Sex steroids can then inhibit or induce the functioning of the hypothalamus and pituitary, forming positive or negative feedback loops. The evidence for circadian control over ovarian function first comes from observation that circadian rhythms of clock gene expression exist in the ovaries of rats and mice (130, 131). For example, LH induces rhythmic gene expression of *Per1* and *Bmal1* in arrhythmic cultured granulosa cells (73). Moreover, both *StAR,* and *Hsd3b* expression follow circadian rhythms in F1 follicles in quail (132) and the *StAR*, and *Dchr7* promoters have functional E-box elements in mice (133). Finally *StAR* mRNA was increased by *Per2* siRNA and unchanged by clock siRNA in ovarian granulosa cells (134), consistent with induction of *StAR* expression by BMAL1 and repression by PER2.

The importance of the ovarian clock for ovarian steroidogenesis in females is emphasized by the observation that *Bmal1*$^{-/-}$ and *Bmal1*$^{fx/fx}$Cre$^{sf\text{-}1}$ females are infertile because of implantation failure due to insufficient ovarian production of progesterone (chapter2). The observation that *StAR* mRNA is reduced in the *Bmal1*$^{-/-}$ (135) and the *Bmal1*$^{fx/fx}$Cre$^{sf\text{-}1}$ females is discussed in chapter2. It is interesting to compare *StAR*$^{-/-}$ and *Bmal1*$^{-/-}$ females. Similar to

Bmal1$^{-/-}$ females, *StAR*$^{-/-}$ mice show reduced progesterone. In contrast to progesterone, estrogen levels appear to be normal in *StAR*$^{-/-}$, *Bmal1*$^{-/-}$ females and *Clock*$^{\Delta 19/\Delta 19}$ females (79, 135, 136)**.** The ovaries of *StAR*$^{-/-}$ females appear normal at birth, and their uteri are indistinguishable from those of wild type females, suggesting existence of *StAR*-independent estrogen production. The vaginas of *StAR*$^{-/-}$ females are open by 8 wk of age (later than *Bmal1*$^{-/-}$ females). Follicular maturation was impaired, corpus lutea were never observed, and females were infertile due to impaired ovulation. By 24 weeks, the *StAR*$^{-/-}$ ovaries are bigger than the wild type ovaries. Furthermore, *StAR*$^{-/-}$ females display symptoms of premature ovarian failure. *Bmal1*$^{-/-}$ females could ovulate and corpus lutea exist in their ovaries although the size is smaller. The differences and overlaps of the phenotype of these two knockouts highlight differences and overlaps in the functions of circadian clock and *StAR*. In summary, the phenotype of *StAR*$^{-/-}$ is more severe than *Bmal1*$^{-/-}$ mice, indicating that although evidence suggest that BMAL1:CLOCK directly control the transcription of *StAR*, there is enough residual *StAR* function in the absence of *StAR* to support virilization in male mice and ovulation in female mcie.

THE REPRODUCTIVE EFFECTS OF CIRCADIAN CLOCKS OUTSIDE OF THE REPRODUCTIVE AXIS.

Reproductive biology can be influenced by a variety of processes that lie outside of the reproductive axis. In order for the best fitness for survival, many organisms integrate permissive signals such as the availability water and food that often can significantly influence whether a pregnancy will continue. Such signals include neuropeptide signaling, leptin signaling, etc.

Neuropeptides

Neuropeptides are small peptides that are produced for interneuronal communication. Neuropeptides play important roles in sensing and integrating a diverse array of information from the body and the environment, and in orchestrating the functioning of the central nervous system and peripheral tissues. In this section I will discuss several neuropeptides, vasopressin, vasoactive intestinal peptide (VIP), oxytocin and neuropeptide Y (NPY), with known roles in reproductive biology, and their interplay with the circadian clock system.

An extensive network of AVP, VIP, oxytocin and NPY fibers is found throughout the central neural system. Neuropeptide Y, VIP and AVP have been implicated in the functioning of the SCN (137). A large body of evidence reveals how circadian clocks function through neuropeptide systems. For example, AVP , VIP, and NPY neurons populate the SCN and these neuropeptides are able to transmit circadian signals within the SCN, and from the SCN to other parts of the brain (138) (Figure 1-7).

Arginine vasopressin. Vasopressin is a neuropeptide produced mainly by the hypothalamus and is stored in pituitary (139). Vasopressin is responsible for regulating water homeostasis in the body. When an organism is dehydrated and plasma volume reduced, osmoreceptors or pressure receptors function to increase the secretion of AVP, which in turn acts on the kidney to retain water for the whole body (140). The role of AVP is well established in food and water intake (141).

Evidence to support a role for circadian biology in AVP regulation comes from the observations that *Avp* mRNA levels in the SCN (114) and AVP protein levels in the cerebrospinal fluid follow circadian rhythms and are often used as an indicator of SCN rhythmicity (54,142). Evidence for a direct circadian-AVP link comes from the observation that

CLOCK: BMAL1 binds to the E-box element of AVP promoter. The importance of AVP for fertility first comes from two lines of investigation. First, Brattleboro rats that have a single base pair deletion in the gene coding for AVP (143, 144). Brattleboro rats display rhythmic locomotor activity in constant darkness (24.6 hours), but body temperature rhythms are altered compared to wild type rats (145). Brattleboro rats display subfertility in both male and females with a reduced rate of conception, a high incidence of fetal deaths, stillbirths, runts, early post-natal deaths, and small litter size. However, mutation of the AVP receptor gene in mice does not result in phenotypes as severe as those seen in Brattleboro rats. Both *Avpr1a*$^{-/-}$ and *Avpr1b*$^{-/-}$ mice are fertile with normal sexual behavior (146, 147), possibly because redundancy exists in the AVP/AVP receptor system. Second, during sexual arousal in men, there is a marked elevation in plasma AVP levels and a return to baseline value by the time of ejaculation. Third, intracerebroventricular injection of AVP on the afternoon of "proestrous day" can rescue the LH surge in *Clock*$^{\Delta 19/\Delta 19}$ females (114), providing direct evidence that circadian clocks regulate LH through AVP.

Oxytocin and AVP are cosynthesized, copackaged in granules, and cosecreted (148). Similar to AVP, oxytocin also follows a circadian rhythm with elevated hormone being released during sleep (148). Oxytocin is reported to play various roles in reproductive biology. In males oxytocin surge from neurohypophysis helps ejaculation by stimulating contractions of the reproductive tract aiding sperm release (149). However, the global oxytocin knockout *Oxt*$^{-/-}$ mice are viable and fertile (150). In females, oxytocin is important for milk production since oxytocin knockout and oxytocin receptor knockout (*Oxtr*$^{-/-}$) female mice fail to produce milk (150). *Oxt*$^{-/-}$ females demonstrate normal maternal behavior, but all offspring die shortly after birth because of the dam's inability to nurse. Postpartum injections of oxytocin to the *Oxt*$^{-/-}$ mothers restore milk

production. In contrast, females in which *Oxtr* is conditionally knocked out in the forebrain ($Oxtr^{fx/fx}Cre^{CamkII}$) can lactate, suggesting a likely cell-autonomous role of mammary *Oxtr* for milk production (151).

Vasoactive intestinal peptide. Vasoactive intestinal peptide (VIP) is a peptide hormone produced in many tissues of vertebrates including the gastrointestinal tract, pancreas and SCN (152). Vasoactive intestinal peptide is present in the gastrointestinal tract, heart, lungs, kidney, urinary bladder, genital organs and the brain (153). Vasoactive intestinal peptide stimulates contractility in the heart, causes vasodilation, lowers arterial blood pressure and relaxes the smooth muscle of trachea (154). VIP-immunoactive fibers are present in SCN, providing nervous control of blood flow.

Vasoactive intestinal peptide, signaling through its receptor *Vipr2*, participates in entrainment to light and maintaining rhythmicity in the SCN (155, 156). Mice null for Vipr2 ($Vipr2^{-/-}$) lack robust circadian rhythms of wheel-running activity and corticosterone secretion, while hepatic clock gene expression is still strongly rhythmic. These $Vipr2^{-/-}$ mice also consume food significantly earlier in the 24 h cycle than wild-type mice (157, 158). The importance of VIP for fertility also comes from global $Vip^{-/-}$ mice. Serum testosterone levels in $Vip^{-/-}$ male mice are lower than those of WT littermates with a reduction of expression of *StAR* and 3β-hydroxysteroid dehydrogenase (3β-Hsd) in the testis. In addition, serum levels of FSH but not LH were reduced in young (159). Second, disruption of *Vipr2*, receptor for VIP also results in defects in male fertility. Aging $Vipr2^{-/-}$ mice (31 week old) exhibit hypospermia and decreased fertility which is accompanied with diffuse seminiferous tubular degeneration (160). In humans VIP injection are used to treat incompetence (161, 162). It is reported that SCN-derived, vasoactive intestinal polypeptide (VIP) secreting neurons project directly to GnRH cells. It is

worth noting that *Vipr2* is also expressed in the testis, epididymis and vas deferens (155), which complicates the effort to conclude that the affected fertility is caused by the specific loss of testicular clock or central clock.

NPY. NPY is a neuropeptide produced mainly by prostate and brain. NPY-immunoreactive fibers are present throughout the rostrocaudal part of the SCN. NPY-highly populated nuclei include the arcuate nucleus, the dorsomedial hypothalamic nucleus and the paraventricular nucleus (Figure 1-7). NPY plays a role as a transmitter from the intergeniculate leaflet (IGL) to the SCN through the geniculohypothalamic tract (GHT) and thus transmit light signals to SCN (163). NPY mimics the effects of light on pineal melatonin and inhibits the effects of light on the induction of *Per1* mRNA (164). Neuropeptide Y is also implicated as mediator between reproduction and energy homeostasis (discussed in the following section). NPY is important for generation of the GnRH surge during proestrus (165). Elevated NPY expression inhibits reproductive function through suppression of GnRH secretion (166). In summary, NPY could be a central neuropeptide coordinator of energy homeostasis, light, and reproduction.

The crosstalk among AVP system, VIP system, NPY system, circadian system and reproductive system provides a good strategy for the organism is adapt to the world with optimal fitness.

Leptin

The hormone leptin is mainly secreted in fat. Leptin plays a key role in a wide range of phenotypes including food intake, body composition, glucose homeostasis, metabolic rate (167). Mutations in leptin or the leptin receptor lead to obesity and diabetes as evidenced by the phenotypes of ob/ob mice and db/db mice respectively. Leptin down-regulates the expression of

Agouti-related peptide (*AgRP*), *NPY* (6) and increases expression of Proopiomelanocortin(*POMC*) (7), suggesting profound regulatory effects on the central nervous system. Cells in the CNS expressing the long isoform of l*epR* have been shown to mediate most of leptin's effects on energy balance (168). This piece of evidence comes from the observation that disruption of *lepR* in peripheral tissues while retaining intact *lepR* in the brain results in no discernable changes in energy balance, thermoregulation, and insulin sensitivity, although hyperleptinemia is present (169). In contrast, deletion of *LepR* in POMC neurons ($LepR^{fx/fx}Cre^{POMC}$) in the arcuate nucleus results in hyperglycemia and obesity (170).

$LepR^{fx/fx}Cre^{Nkx2.1}$ mice , in which *LepR* is knocked out in the hypothalamus but not in more caudal brain regions, display increased weight gain and adiposity, hyperphagia, cold intolerance, and insulin resistance from weaning until 8 weeks of age. However, after 8 weeks of age, $LepR^{fx/fx}Cre^{Nkx2.1}$ mice maintained stable adiposity levels. In contrast, the body fat percentage of db/db mice continues to escalate with age, suggesting that remaining leptin signals in $LepR^{fx/fx}Cre^{Nkx2.1}$ mice mediate physiological adaptations that prevent the escalation of the adiposity phenotype in adult mice. The persistence of severe adiposity in $LepR^{fx/fx}Cre^{Nkx2.1}$ mice, however, suggests that compensatory actions of circuits regulating growth and energy expenditure are not sufficient to reverse obesity established at an early age.

The evidence that the leptin/leptin signaling system is under circadian control comes from the following observations. Serum leptin levels follow a diurnal rhythm (171). Leptin's action is mediated by binding to the long isoform of the leptin receptor (*LepR*) present in the hypothalamus and activating signaling cascades of different pathways including the JAK-STAT pathway. Hypothalamic *LepR* gene expression also follows a circadian rhythm (172). There are putative E-box elements in the promoter of leptin gene (173). Managing the energy system

according to the solar system in circadian rhythms obviously put organisms at a survival advantage.

It is hypothesized that leptin signalling system could provide a permissive signal of energy homeostasis for fertility. The leptin/lepR signalling deficiency results in infertility as evidenced by the infertility of leptin deficient ob/ob mice and *LepR* deficient db/db mice (174). ob/ob males have lower FSH and testosterone levels, smaller testis and seminal vesicles than lean mice. Diet restriction can restore fertility of some ob/ob male, but not female mice. Ob/ob females display acyclic estrous cycles (persistent estrus or persistent metestrus) (175), smaller ovaries, underdeveloped uteri, and mammary glands with big fat pads. Reconstitution of reproductive functions in the ob/ob female necessitates delivery of hypothalamic extracts to the third ventricle and administration of pituitary extracts, gonadotropic hormones, progesterone and relaxin. These findings demonstrate that the sterility of ob/ob females is caused by an insufficiency of hormones rather than physical hindrance of copulatory activity, pregnancy and parturition caused by excess adipose tissue.

There are many studies on how leptin regulates reproduction. Comparing the reproductive phenotype among conditional *LepR* knockout mice in different neurons shows that the regulation of fertility is not through direct action on GnRH neurons since LepR$b^{fx/fx}Cre^{GnRH}$ mice are fertile. In contrast, Quennel reported that forebrain neuronal *LepR* deletion (LepR$b^{fx/fx}Cre^{CamkII}$) caused reduced fertility and late onset of puberty (176).

Kisspeptin neurons express leptin receptors, and the kisspeptin system may participate in transmitting metabolic information to the GnRH neurons, thus providing a bridge between metabolic regulation and fertility (177).

NPY has been implicated in the regulation of energy balance and reproduction, and chronically elevated NPY levels in the hypothalamus are associated with obesity and reduced reproductive function. Crossing the NPY receptor knockout mouse ($Y4^{-/-}$) onto the *ob/ob* background upregulated testosterone levels, and restored testis and seminal vesicle size and morphology to that of wild type controls (178). Treatment of *ob/ob* mice with leptin reduces NPY mRNA expression and peptide levels in the hypothalamus, reduces the hyperphagic, obese phenotype, and also restores fertility of male and female mice (167).

Ob/ob or db/db mice share phenotypes with circadian gene mutants. First, ob/ob, $Clock^{\Delta 19/\Delta 19}$ and $Bmal1^{-/-}$ mice have diabetes (179, 180). $Clock^{\Delta 19/\Delta 19}$ mice are hyperphagic and obese, and develop a metabolic syndrome of hyperleptinemia, hyperlipidemia, hyperglycemia, and hypoinsulinemia (181). Second, the circadian patterns of eating and drinking of ob/ob mice differed from those of lean mice, both temporally and with respect to the absolute amounts consumed hourly (182). $Clock^{\Delta 19/\Delta 19}$ *and* $Bmal1^{-/-}$ mice also have a greatly attenuated diurnal feeding rhythm.

In chapter 3, I will discuss the phenotype of central nervous system conditional knockout *Bmal1* ($Bmal1^{fx/fx}Cre^{nestin}$). Briefly, like ob/ob mice, $Bmal1^{fx/fx}Cre^{nestin}$ males have decreased fertility, smaller testis and seminal vesicles. Furthermore, those infertile $Bmal1^{fx/fx}Cre^{nestin}$ males that are infertile are also obese.

It appears that conditional knockouts of the leptin signaling and circadian clock systems share some phenotypes. $LepR^{fx/fx}Cre^{CamkII}$ males (forebrain deletion) have reduced fertility, so do $Bmal1^{fx/fx}Cre^{Nestin}$ males. $LepR^{fx/fx}Cre^{sf\text{-}1}$ and $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ males are all fertile, indicating that disruption of the peripheral *LepR* or *Bmal1* in the reproductive axis is not enough to affect the functions for male fertility. It would be interesting to observe the phenotypes of conditional

knockouts such as *Bmal1*$^{fx/fx}$*Cre*^{GnRH}, *Bmal1*$^{fx/fx}$*Cre*^{CamkII} *and Bmal1*$^{fx/fx}$*Cre*^{LepRb}, and do more detailed comparisons.

Aromatase

Aromatase is expressed in cells within the developing SCN, retina, optic tract, and geniculate bodies, including the intrageniculate leaflet (IGL) (183). The E-box elements exists in the promoter region of the bovine aromatase gene (134).

Aromatase$^{-/-}$ males showed impaired mounting behavior and are subfertile (184). The absence of an odor preference in *Aromatase*$^{-/-}$ males when provided with volatile odors suggests that aromatization of testosterone to estradiol is required for normal functioning of the male olfactory system.

SCN

The SCN not only regulates circadian rhythm, but also regulates neuroendocrine functions. Male hamsters and rats show a diurnal rhythm of sexual performance displayed by increased speed and efficacy of copulation during dark phase as compared to the light phase in a daily light-dark cycle. Destruction of the suprachiasmatic nuclei (SCN) eliminates this normal diurnal rhythm of sexual behavior (185).

Recent evidence suggests an organizing effect of steroid hormones in the SCN. In adult mammals, the SCN synthesizes estrogen, progesterone and testosterone receptors (76). StAR, aromatase, 17β hsd, 5α reductase, 3βhsd, 11β hsd, p450c11b and aldosterone synthase are all expressed in brain nervous system at the mRNA, and protein levels. It is possible that not only does SCN regulate the reproductive system, the reproductive system could in turn regulate the functioning of SCN (Figure 1-7).

Neonatal exposure to hormones may be critical for the organization of the brain. Males are exposed to higher levels of testosterone when they develop between two males than between females in utero (186). These differences could alter their traits after birth.

Another example comes from the effect of neonatal exposure to leptin. Offspring of low-protein fed dams have increased susceptibility to diabetes, and insulin resistance when fed a high-fat diet. Administration of leptin during pregnancy and lactation to these protein-restricted dams produces offspring that have increased metabolic rate and do not become obese or insulin (187).

Olfactory Bulb

The brain contains multiple oscillators outside the SCN. In addition to POA areas mentioned above, the olfactory bulb is another region that has been suggested to contain a self-sustaining circadian oscillator (188) (189). *Per1, Per2, Bmal1* follow a circadian expression in the olfactory bulb (188). Given that smelling is the common step of social contact between male mice and female mice, and pheromones play important roles in rodent sexual behavior, it is possible that biological functional implications exist behind the rhythmic expression of circadian clocks in olfactory bulb. The lesion of the olfactory bulbs in adult female mice led to reduced ovarian weight and absence of corpora, suggesting roles of olfactory bulb in female fertility (190).

CONCLUSION

Circadian regulation of reproductive biology exist in all cell types in the reproductive axis, SCN and extra-SCN regions in the brain. Circadian genes can profoundly regulate steroidogenesis, energy balance, aging processes and behavior. These various aspects could

form complex interaction loops, thus optimizing the fitness of the organism to the 24-hour rhythm of light and day in this solar system.

REFRENCE

1. Karatsoreos IN, Bhagat S, Bloss EB, Morrison JH, & McEwen BS (2011) Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc Natl Acad Sci U S A* 108(4):1657-1662.
2. Wollnik F (1989) Physiology and regulation of biological rhythms in laboratory animals: an overview. *Lab Anim* 23(2):107-125.
3. Klingman KM, Marsh EE, Klerman EB, Anderson EJ, & Hall JE (2011) Absence of circadian rhythms of gonadotropin secretion in women. *J Clin Endocrinol Metab* 96(5):1456-1461.
4. Baird AL, Coogan AN, Siddiqui A, Donev RM, & Thome J (2011) Adult attention-deficit hyperactivity disorder is associated with alterations in circadian rhythms at the behavioural, endocrine and molecular levels. *Mol Psychiatry*.
5. Hoshino K, Wakatsuki Y, Iigo M, & Shibata S (2006) Circadian Clock mutation in dams disrupts nursing behavior and growth of pups. *Endocrinology* 147(4):1916-1923.
6. Fu L, Patel MS, & Karsenty G (2006) The circadian modulation of leptin-controlled bone formation. *Prog Brain Res* 153:177-188.
7. Medina D*, et al.* (2001) Mechanisms of hormonal prevention of breast cancer. *Ann N Y Acad Sci* 952:23-35.
8. Chiaverotti TA & Drinkwater NR (2003) C57BR/cdJ hepatocarcinogen susceptibility genes act cell-autonomously in C57BR/cdJ<-->C57BL/6J chimeras. *Cancer Res* 63(16):4914-4919.
9. Moser AR, Pitot HC, & Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 247(4940):322-324.
10. Robinson SP & Jordan VC (1987) Reversal of the antitumor effects of tamoxifen by progesterone in the 7,12-dimethylbenzanthracene-induced rat mammary carcinoma model. *Cancer Res* 47(20):5386-5390.
11. Bugni JM, Poole TM, & Drinkwater NR (2001) The little mutation suppresses DEN-induced hepatocarcinogenesis in mice and abrogates genetic and hormonal modulation of susceptibility. *Carcinogenesis* 22(11):1853-1862.
12. Giroux V, Lemay F, Bernatchez G, Robitaille Y, & Carrier JC (2008) Estrogen receptor beta deficiency enhances small intestinal tumorigenesis in ApcMin/+ mice. *Int J Cancer* 123(2):303-311.
13. Van Cauter E, Spiegel K, Tasali E, & Leproult R (2008) Metabolic consequences of sleep and sleep loss. *Sleep Med* 9 Suppl 1:S23-28.
14. Beccuti G & Pannain S (2011) Sleep and obesity. *Curr Opin Clin Nutr Metab Care* 14(4):402-412.
15. Spiegel K, Tasali E, Leproult R, & Van Cauter E (2009) Effects of poor and short sleep on glucose metabolism and obesity risk. *Nat Rev Endocrinol* 5(5):253-261.
16. Schernhammer ES*, et al.* (2003) Night-shift work and risk of colorectal cancer in the nurses' health study. *J Natl Cancer Inst* 95(11):825-828.
17. Lie JA*, et al.* (2011) Night work and breast cancer risk among Norwegian nurses: assessment by different exposure metrics. *Am J Epidemiol* 173(11):1272-1279.
18. Kubo T*, et al.* (2006) Prospective cohort study of the risk of prostate cancer among rotating-shift workers: findings from the Japan collaborative cohort study. *Am J Epidemiol* 164(6):549-555.

19. Nievergelt CM*, et al.* (2006) Suggestive evidence for association of the circadian genes PERIOD3 and ARNTL with bipolar disorder. *Am J Med Genet B Neuropsychiatr Genet* 141B(3):234-241.
20. Woon PY*, et al.* (2007) Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc Natl Acad Sci U S A* 104(36):14412-14417.
21. Woon PY*, et al.* (2007) Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc Natl Acad Sci U S A* 104(36):14412-14417.
22. Zhu Y*, et al.* (2009) Testing the circadian gene hypothesis in prostate cancer: a population-based case-control study. *Cancer Res* 69(24):9315-9322.
23. Scott EM, Carter AM, & Grant PJ (2008) Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. *Int J Obes (Lond)* 32(4):658-662.
24. Dai H*, et al.* (2011) The role of polymorphisms in circadian pathway genes in breast tumorigenesis. *Breast Cancer Res Treat* 127(2):531-540.
25. Sookoian S, Castaño G, Gemma C, Gianotti TF, & Pirola CJ (2007) Common genetic variations in CLOCK transcription factor are associated with nonalcoholic fatty liver disease. *World J Gastroenterol* 13(31):4242-4248.
26. Garaulet M*, et al.* (2010) PERIOD2 variants are associated with abdominal obesity, psycho-behavioral factors, and attrition in the dietary treatment of obesity. *J Am Diet Assoc* 110(6):917-921.
27. Dallaspezia S*, et al.* (2011) Circadian clock gene Per3 variants influence the postpartum onset of bipolar disorder. *Eur Psychiatry* 26(3):138-140.
28. Smith JF*, et al.* (2009) Sexual, marital, and social impact of a man's perceived infertility diagnosis. *J Sex Med* 6(9):2505-2515.
29. Stephen EH & Chandra A (1998) Updated projections of infertility in the United States: 1995-2025. *Fertil Steril* 70(1):30-34.
30. Achermann JC & Jameson JL (1999) Fertility and infertility: genetic contributions from the hypothalamic-pituitary-gonadal axis. *Mol Endocrinol* 13(6):812-818.
31. Labyak S, Lava S, Turek F, & Zee P (2002) Effects of shiftwork on sleep and menstrual function in nurses. *Health Care Women Int* 23(6-7):703-714.
32. Kovanen L, Saarikoski ST, Aromaa A, Lonnqvist J, & Partonen T (2010) ARNTL (BMAL1) and NPAS2 gene variants contribute to fertility and seasonality. *PLoS One* 5(4):e10007.
33. Di Lorenzo L*, et al.* (2003) Effect of shift work on body mass index: results of a study performed in 319 glucose-tolerant men working in a Southern Italian industry. *Int J Obes Relat Metab Disord* 27(11):1353-1358.
34. Feldman HA*, et al.* (2000) Erectile dysfunction and coronary risk factors: prospective results from the Massachusetts male aging study. *Prev Med* 30(4):328-338.
35. Pasqualotto FF, Borges Junior E, & Pasqualotto EB (2008) The male biological clock is ticking: a review of the literature. *Sao Paulo Med J* 126(3):197-201.
36. Lambert SM, Masson P, & Fisch H (2006) The male biological clock. *World J Urol* 24(6):611-617.
37. Monk TH (2000) What can the chronobiologist do to help the shift worker? *J Biol Rhythms* 15(2):86-94.
38. Kulikov VY, Fridman YM, & Fomin AN (2007) Role of oxidative stress in mechanisms of premature aging in shift labor workers. *Alaska Med* 49(2 Suppl):81-84.
39. Lehman MN*, et al.* (1987) Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *J Neurosci* 7(6):1626-1638.

40. Shi S*, et al.* (2010) Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. *Curr Biol* 20(4):316-321.
41. Westermark PO, Welsh DK, Okamura H, & Herzel H (2009) Quantification of circadian rhythms in single cells. *PLoS Comput Biol* 5(11):e1000580.
42. Bouskila Y & Dudek FE (1995) Can a population of suprachiasmatic nucleus neurons with different period lengths produce a stable circadian rhythm? *Brain Res* 670(2):333-336.
43. Nakamura TJ*, et al.* (2010) Influence of the estrous cycle on clock gene expression in reproductive tissues: effects of fluctuating ovarian steroid hormone levels. *Steroids* 75(3):203-212.
44. Yoo SH*, et al.* (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A* 101(15):5339-5346.
45. Maywood ES, O'Neill JS, Chesham JE, & Hastings MH (2007) Minireview: The circadian clockwork of the suprachiasmatic nuclei--analysis of a cellular oscillator that drives endocrine rhythms. *Endocrinology* 148(12):5624-5634.
46. Boden MJ & Kennaway DJ (2006) Circadian rhythms and reproduction. *Reproduction* 132(3):379-392.
47. Dunlap JC (1999) Molecular bases for circadian clocks. *Cell* 96(2):271-290.
48. Gekakis N*, et al.* (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* 280(5369):1564-1569.
49. Guillaumond F, Dardente H, Giguère V, & Cermakian N (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* 20(5):391-403.
50. Yamajuku D*, et al.* (2011) Cellular DBP and E4BP4 proteins are critical for determining the period length of the circadian oscillator. *FEBS Lett* 585(14):2217-2222.
51. Emery P & Reppert SM (2004) A rhythmic Ror. *Neuron* 43(4):443-446.
52. Ueda HR*, et al.* (2005) System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat Genet* 37(2):187-192.
53. Hogenesch JB, Panda S, Kay S, & Takahashi JS (2003) Circadian transcriptional output in the SCN and liver of the mouse. *Novartis Found Symp* 253:171-180; discussion 152-175, 102-179, 180-173 passim.
54. van Esseveldt KE, van der Geest BA, Duindam H, Rietveld WJ, & Boer GJ (1999) Circadian rhythmicity of vasopressin levels in the cerebrospinal fluid of suprachiasmatic nucleus-lesioned and -grafted rats. *J Biol Rhythms* 14(1):28-36.
55. Son GH*, et al.* (2008) Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci U S A* 105(52):20970-20975.
56. Leclerc GM & Boockfor FR (2005) Pulses of prolactin promoter activity depend on a noncanonical E-box that can bind the circadian proteins CLOCK and BMAL1. *Endocrinology* 146(6):2782-2790.
57. Matsuo T*, et al.* (2003) Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 302(5643):255-259.
58. Boden MJ, Varcoe TJ, Voultsios A, & Kennaway DJ (2010) Reproductive biology of female Bmal1 null mice. *Reproduction* 139(6):1077-1090.
59. Bunger MK*, et al.* (2005) Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis* 41(3):122-132.
60. Chen Y, Xu X, Tan Z, Ye C, & Zhao Q (2012) Age-related BMAL1 change affects mouse bone marrow stromal cell proliferation and osteo-differentiation potential. *Arch Med Sci* 8(1):30-38.

61. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, & Antoch MP (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* 20(14):1868-1873.
62. Marcheva B*, et al.* (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* 466(7306):627-631.
63. Hu Y*, et al.* (2011) Selenium is a modulator of circadian clock that protects mice from the toxicity of a chemotherapeutic drug via upregulation of the core clock protein, BMAL1. *Oncotarget* 2(12):1279-1290.
64. Bunger MK*, et al.* (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* 103(7):1009-1017.
65. Hogenesch JB*, et al.* (2000) The basic helix-loop-helix-PAS protein MOP9 is a brain-specific heterodimeric partner of circadian and hypoxia factors. *J Neurosci* 20(13):RC83.
66. Yu EA & Weaver DR (2011) Disrupting the circadian clock: gene-specific effects on aging, cancer, and other phenotypes. *Aging (Albany NY)* 3(5):479-493.
67. Debruyne JP*, et al.* (2006) A clock shock: mouse CLOCK is not required for circadian oscillator function. *Neuron* 50(3):465-477.
68. Bae K*, et al.* (2001) Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron* 30(2):525-536.
69. van der Horst GT*, et al.* (1999) Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature* 398(6728):627-630.
70. DeBruyne JP, Weaver DR, & Reppert SM (2007) CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nat Neurosci* 10(5):543-545.
71. DeBruyne JP, Weaver DR, & Reppert SM (2007) Peripheral circadian oscillators require CLOCK. *Curr Biol* 17(14):R538-539.
72. Shi S*, et al.* (2010) Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. *Curr Biol* 20(4):316-321.
73. Tischkau SA, Howell RE, Hickok JR, Krager SL, & Bahr JM (2011) The luteinizing hormone surge regulates circadian clock gene expression in the chicken ovary. *Chronobiol Int* 28(1):10-20.
74. Meites J (1990) Aging: hypothalamic catecholamines, neuroendocrine-immune interactions, and dietary restriction. *Proc Soc Exp Biol Med* 195(3):304-311.
75. Karatsoreos IN & Silver R (2007) Minireview: The neuroendocrinology of the suprachiasmatic nucleus as a conductor of body time in mammals. *Endocrinology* 148(12):5640-5647.
76. Shughrue PJ, Lane MV, & Merchenthaler I (1997) Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J Comp Neurol* 388(4):507-525.
77. Alvarez JD*, et al.* (2008) The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J Biol Rhythms* 23(1):26-36.
78. Bertolucci C*, et al.* (2008) Evidence for an overlapping role of CLOCK and NPAS2 transcription factors in liver circadian oscillators. *Mol Cell Biol* 28(9):3070-3075.
79. Miller BH*, et al.* (2004) Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. *Curr Biol* 14(15):1367-1373.
80. Pilorz V & Steinlechner S (2008) Low reproductive success in Per1 and Per2 mutant mouse females due to accelerated ageing? *Reproduction* 135(4):559-568.
81. Harman SM & Talbert GB (1970) The effect of maternal age on ovulation, corpora lutea of pregnancy, and implantation failure in mice. *J Reprod Fertil* 23(1):33-39.
82. Tobet SA, Bless EP, & Schwarting GA (2001) Developmental aspect of the gonadotropin-releasing hormone system. *Mol Cell Endocrinol* 185(1-2):173-184.
83. Hermann M, Untergasser G, Rumpold H, & Berger P (2000) Aging of the male reproductive system. *Exp Gerontol* 35(9-10):1267-1279.

84. Rao CV & Lei ZM (2002) Consequences of targeted inactivation of LH receptors. *Mol Cell Endocrinol* 187(1-2):57-67.
85. Franz WB (1988) Basic review: endocrinology of the normal menstrual cycle. *Prim Care* 15(3):607-616.
86. Tarozzo G*, et al.* (1995) Development and migration of olfactory neurones in the nervous system of the neonatal opossum. *Proc Biol Sci* 262(1363):95-101.
87. Hickok JR & Tischkau SA (2010) In vivo circadian rhythms in gonadotropin-releasing hormone neurons. *Neuroendocrinology* 91(1):110-120.
88. Funabashi T, Shinohara K, Mitsushima D, & Kimura F (2000) Gonadotropin-releasing hormone exhibits circadian rhythm in phase with arginine-vasopressin in co-cultures of the female rat preoptic area and suprachiasmatic nucleus. *J Neuroendocrinol* 12(6):521-528.
89. Jin X*, et al.* (1999) A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 96(1):57-68.
90. Matagne V*, et al.* (2012) TTF1, a homeodomain containing transcription factor, contributes to regulating periodic oscillations in GnRH gene expression. *J Neuroendocrinol*.
91. Matagne V*, et al.* (2012) TTF1, a homeodomain containing transcription factor, contributes to regulating periodic oscillations in GnRH gene expression. *J Neuroendocrinol*.
92. Mason AJ*, et al.* (1986) The hypogonadal mouse: reproductive functions restored by gene therapy. *Science* 234(4782):1372-1378.
93. Livne I, Silverman AJ, & Gibson MJ (1992) Reversal of reproductive deficiency in the hpg male mouse by neonatal androgenization. *Biol Reprod* 47(4):561-567.
94. Gibson MJ, Kokoris GJ, & Silverman AJ (1988) Positive feedback in hypogonadal female mice with preoptic area brain transplants. *Neuroendocrinology* 48(2):112-119.
95. Antz-Vaxman M & Aron C (1986) Olfactory environment and coitus-induced ovulation and/or luteinization in the cyclic female rat. *Biol Reprod* 34(1):237-243.
96. Arai Y, Murakami S, & Nishizuka M (1994) Androgen enhances neuronal degeneration in the developing preoptic area: apoptosis in the anteroventral periventricular nucleus (AVPvN-POA). *Horm Behav* 28(4):313-319.
97. Roa J, Navarro VM, & Tena-Sempere M (2011) Kisspeptins in reproductive biology: consensus knowledge and recent developments. *Biol Reprod* 85(4):650-660.
98. Lapatto R*, et al.* (2007) Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. *Endocrinology* 148(10):4927-4936.
99. Kauffman AS*, et al.* (2007) The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior. *J Neurosci* 27(33):8826-8835.
100. Williams WP, 3rd, Jarjisian SG, Mikkelsen JD, & Kriegsfeld LJ (2011) Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology* 152(2):595-606.
101. Lapatto R*, et al.* (2007) Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. *Endocrinology* 148(10):4927-4936.
102. Mayer C & Boehm U (2011) Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nat Neurosci* 14(6):704-710.
103. Takahashi Y, Kipnis DM, & Daughaday WH (1968) Growth hormone secretion during sleep. *J Clin Invest* 47(9):2079-2090.
104. Sassin JF, Frantz AG, Weitzman ED, & Kapen S (1972) Human prolactin: 24-hour pattern with increased release during sleep. *Science* 177(4055):1205-1207.
105. Weitzman ED, Schaumburg H, & Fishbein W (1966) Plasma 17-hydroxycorticosteroid levels during sleep in man. *J Clin Endocrinol Metab* 26(2):121-127.

106. Mitamura R*, et al.* (2000) Diurnal rhythms of luteinizing hormone, follicle-stimulating hormone, testosterone, and estradiol secretion before the onset of female puberty in short children. *J Clin Endocrinol Metab* 85(3):1074-1080.
107. Schang AL*, et al.* (2012) Identification and Analysis of Two Novel Sites of Rat GnRH Receptor Gene Promoter Activity: The Pineal Gland and Retina. *Neuroendocrinology*.
108. Resuehr D, Wildemann U, Sikes H, & Olcese J (2007) E-box regulation of gonadotropin-releasing hormone (GnRH) receptor expression in immortalized gonadotrope cells. *Mol Cell Endocrinol* 278(1-2):36-43.
109. Mitamura R*, et al.* (1999) Diurnal rhythms of luteinizing hormone, follicle-stimulating hormone, and testosterone secretion before the onset of male puberty. *J Clin Endocrinol Metab* 84(1):29-37.
110. Jean-Faucher C, Berger M, de Turckheim M, Veyssiere G, & Jean C (1986) Circadian variations in plasma LH and FSH in juvenile and adult male mice. *Horm Res* 23(3):185-192.
111. Coquelin A, Clancy AN, Macrides F, Noble EP, & Gorski RA (1984) Pheromonally induced release of luteinizing hormone in male mice: involvement of the vomeronasal system. *J Neurosci* 4(9):2230-2236.
112. Richards JS (2005) Ovulation: new factors that prepare the oocyte for fertilization. *Mol Cell Endocrinol* 234(1-2):75-79.
113. Smith MS, Freeman ME, & Neill JD (1975) The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology* 96(1):219-226.
114. Miller BH*, et al.* (2006) Vasopressin regulation of the proestrous luteinizing hormone surge in wild-type and Clock mutant mice. *Biol Reprod* 75(5):778-784.
115. Van der Beek EM, Horvath TL, Wiegant VM, Van den Hurk R, & Buijs RM (1997) Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotropin-releasing hormone system: combined tracing and light and electron microscopic immunocytochemical studies. *J Comp Neurol* 384(4):569-579.
116. Bole-Feysot C, Goffin V, Edery M, Binart N, & Kelly PA (1998) Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 19(3):225-268.
117. Ormandy CJ*, et al.* (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* 11(2):167-178.
118. Laron Z & Klinger B (1998) Effect of insulin-like growth factor-I treatment on serum androgens and testicular and penile size in males with Laron syndrome (primary growth hormone resistance). *Eur J Endocrinol* 138(2):176-180.
119. Spiliotis BE (2003) Growth hormone insufficiency and its impact on ovarian function. *Ann N Y Acad Sci* 997:77-84.
120. de Boer JA, Schoemaker J, & van der Veen EA (1997) Impaired reproductive function in women treated for growth hormone deficiency during childhood. *Clin Endocrinol (Oxf)* 46(6):681-689.
121. Bartke A (1966) Reproduction of female dwarf mice treated with prolactin. *J Reprod Fertil* 11(2):203-206.
122. Bartke A (1999) Role of growth hormone and prolactin in the control of reproduction: what are we learning from transgenic and knock-out animals? *Steroids* 64(9):598-604.
123. Sellix MT*, et al.* (2006) Anatomical and functional characterization of clock gene expression in neuroendocrine dopaminergic neurons. *Am J Physiol Regul Integr Comp Physiol* 290(5):R1309-1323.
124. Palm IF*, et al.* (2001) Control of the estradiol-induced prolactin surge by the suprachiasmatic nucleus. *Endocrinology* 142(6):2296-2302.

125. Coquelin A & Desjardins C (1982) Luteinizing hormone and testosterone secretion in young and old male mice. *Am J Physiol* 243(3):E257-263.
126. Hagenauer MH & Lee TM (2011) Time for testosterone: the suprachiasmatic nucleus gets sexy. *Endocrinology* 152(5):1727-1730.
127. Mattern LG, Helmreich DL, & Cameron JL (1993) Diurnal pattern of pulsatile luteinizing hormone and testosterone secretion in adult male rhesus monkeys (Macaca mulatta): influence of the timing of daily meal intake. *Endocrinology* 132(3):1044-1054.
128. Bittman EL, Doherty L, Huang L, & Paroskie A (2003) Period gene expression in mouse endocrine tissues. *Am J Physiol Regul Integr Comp Physiol* 285(3):R561-569.
129. Morse D, Cermakian N, Brancorsini S, Parvinen M, & Sassone-Corsi P (2003) No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. *Mol Endocrinol* 17(1):141-151.
130. Fahrenkrug J, Georg B, Hannibal J, Hindersson P, & Gras S (2006) Diurnal rhythmicity of the clock genes Per1 and Per2 in the rat ovary. *Endocrinology* 147(8):3769-3776.
131. Dolatshad H, Davis FC, & Johnson MH (2009) Circadian clock genes in reproductive tissues and the developing conceptus. *Reprod Fertil Dev* 21(1):1-9.
132. Nakao N*, et al.* (2007) Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. *Endocrinology* 148(7):3031-3038.
133. Kim JH, Lee JN, & Paik YK (2001) Cholesterol biosynthesis from lanosterol. A concerted role for Sp1 and NF-Y-binding sites for sterol-mediated regulation of rat 7-dehydrocholesterol reductase gene expression. *J Biol Chem* 276(21):18153-18160.
134. Shimizu T*, et al.* (2011) Circadian Clock genes Per2 and clock regulate steroid production, cell proliferation, and luteinizing hormone receptor transcription in ovarian granulosa cells. *Biochem Biophys Res Commun* 412(1):132-135.
135. Ratajczak CK, Boehle KL, & Muglia LJ (2009) Impaired steroidogenesis and implantation failure in Bmal1-/- mice. *Endocrinology* 150(4):1879-1885.
136. Ishii T*, et al.* (2002) The roles of circulating high-density lipoproteins and trophic hormones in the phenotype of knockout mice lacking the steroidogenic acute regulatory protein. *Mol Endocrinol* 16(10):2297-2309.
137. Reghunandanan V & Reghunandanan R (2006) Neurotransmitters of the suprachiasmatic nuclei. *J Circadian Rhythms* 4:2.
138. Kalsbeek A & Buijs RM (2002) Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell Tissue Res* 309(1):109-118.
139. Hertz L, Chen Y, & Spatz M (2000) Involvement of non-neuronal brain cells in AVP-mediated regulation of water space at the cellular, organ, and whole-body level. *J Neurosci Res* 62(4):480-490.
140. Nielsen S*, et al.* (1995) Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc Natl Acad Sci U S A* 92(4):1013-1017.
141. Racotta R, Soto-Mora LM, Palacios E, & Quevedo L (1995) Norepinephrine inhibition of water and food intake: comparison with vasopressin effects. *Physiol Behav* 57(1):141-145.
142. Gimpl G & Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* 81(2):629-683.
143. Boer K, Boer GJ, & Swaab DF (1981) Reproduction in Brattleboro rats with diabetes insipidus. *J Reprod Fertil* 61(2):273-280.
144. Birkett SD & Pickering BT (1988) The vasopressin precursor in the Brattleboro (di/di) rat. *Int J Pept Protein Res* 32(6):565-572.

145. Wideman CH, Murphy HM, & Nadzam GR (2000) Vasopressin deficiency provides evidence for separate circadian oscillators of activity and temperature. *Peptides* 21(6):811-816.
146. Wersinger SR, Caldwell HK, Christiansen M, & Young WS (2007) Disruption of the vasopressin 1b receptor gene impairs the attack component of aggressive behavior in mice. *Genes Brain Behav* 6(7):653-660.
147. Wersinger SR*, et al.* (2007) Vasopressin 1a receptor knockout mice have a subtle olfactory deficit but normal aggression. *Genes Brain Behav* 6(6):540-551.
148. Forsling ML (2000) Diurnal rhythms in neurohypophysial function. *Exp Physiol* 85 Spec No:179S-186S.
149. Thackare H, Nicholson HD, & Whittington K (2006) Oxytocin--its role in male reproduction and new potential therapeutic uses. *Hum Reprod Update* 12(4):437-448.
150. Nishimori K*, et al.* (1996) Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. *Proc Natl Acad Sci U S A* 93(21):11699-11704.
151. Lee HJ, Caldwell HK, Macbeth AH, Tolu SG, & Young WS (2008) A conditional knockout mouse line of the oxytocin receptor. *Endocrinology* 149(7):3256-3263.
152. Fahrenkrug J (2001) Gut/brain peptides in the genital tract: VIP and PACAP. *Scand J Clin Lab Invest Suppl* 234:35-39.
153. Moody TW, Ito T, Osefo N, & Jensen RT (2011) VIP and PACAP: recent insights into their functions/roles in physiology and disease from molecular and genetic studies. *Curr Opin Endocrinol Diabetes Obes* 18(1):61-67.
154. Henning RJ & Sawmiller DR (2001) Vasoactive intestinal peptide: cardiovascular effects. *Cardiovasc Res* 49(1):27-37.
155. Harmar AJ*, et al.* (2004) Distribution of the VPAC2 receptor in peripheral tissues of the mouse. *Endocrinology* 145(3):1203-1210.
156. Harmar AJ*, et al.* (2002) The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell* 109(4):497-508.
157. Sheward WJ*, et al.* (2007) Entrainment to feeding but not to light: circadian phenotype of VPAC2 receptor-null mice. *J Neurosci* 27(16):4351-4358.
158. Cutler DJ*, et al.* (2003) The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro. *Eur J Neurosci* 17(2):197-204.
159. Lacombe A*, et al.* (2007) Lack of vasoactive intestinal peptide reduces testosterone levels and reproductive aging in mouse testis. *J Endocrinol* 194(1):153-160.
160. Asnicar MA*, et al.* (2002) Vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 2 deficiency in mice results in growth retardation and increased basal metabolic rate. *Endocrinology* 143(10):3994-4006.
161. Dinsmore WW*, et al.* (1999) Treating men with predominantly nonpsychogenic erectile dysfunction with intracavernosal vasoactive intestinal polypeptide and phentolamine mesylate in a novel auto-injector system: a multicentre double-blind placebo-controlled study. *BJU Int* 83(3):274-279.
162. Sandhu D*, et al.* (1999) A double blind, placebo controlled study of intracavernosal vasoactive intestinal polypeptide and phenotolamine mesylate in a novel auto-injector for the treatment of non-psychogenic erectile dysfunction. *Int J Impot Res* 11(2):91-97.
163. Goel N, Lee TM, & Smale L (1999) Suprachiasmatic nucleus and intergeniculate leaflet in the diurnal rodent Octodon degus: retinal projections and immunocytochemical characterization. *Neuroscience* 92(4):1491-1509.
164. Gamble KL, Paul KN, Karom MC, Tosini G, & Albers HE (2006) Paradoxical effects of NPY in the suprachiasmatic nucleus. *Eur J Neurosci* 23(9):2488-2494.

165. Leupen SM, Besecke LM, & Levine JE (1997) Neuropeptide Y Y1-receptor stimulation is required for physiological amplification of preovulatory luteinizing hormone surges. *Endocrinology* 138(7):2735-2739.
166. Pierroz DD, Gruaz NM, d'Alieves V, & Aubert ML (1995) Chronic administration of neuropeptide Y into the lateral ventricle starting at 30 days of life delays sexual maturation in the female rat. *Neuroendocrinology* 61(3):293-300.
167. Chehab FF, Qiu J, & Ogus S (2004) The use of animal models to dissect the biology of leptin. *Recent Prog Horm Res* 59:245-266.
168. Ring LE & Zeltser LM (2010) Disruption of hypothalamic leptin signaling in mice leads to early-onset obesity, but physiological adaptations in mature animals stabilize adiposity levels. *J Clin Invest* 120(8):2931-2941.
169. Guo K*, et al.* (2007) Disruption of peripheral leptin signaling in mice results in hyperleptinemia without associated metabolic abnormalities. *Endocrinology* 148(8):3987-3997.
170. Berglund ED*, et al.* (2012) Direct leptin action on POMC neurons regulates glucose homeostasis and hepatic insulin sensitivity in mice. *J Clin Invest* 122(3):1000-1009.
171. Ahima RS*, et al.* (1996) Role of leptin in the neuroendocrine response to fasting. *Nature* 382(6588):250-252.
172. Stutz AM, Staszkiewicz J, Ptitsyn A, & Argyropoulos G (2007) Circadian expression of genes regulating food intake. *Obesity (Silver Spring)* 15(3):607-615.
173. Mason MM, He Y, Chen H, Quon MJ, & Reitman M (1998) Regulation of leptin promoter function by Sp1, C/EBP, and a novel factor. *Endocrinology* 139(3):1013-1022.
174. Chehab FF, Lim ME, & Lu R (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 12(3):318-320.
175. Ng KY, Yong J, & Chakraborty TR (2010) Estrous cycle in ob/ob and ovariectomized female mice and its relation with estrogen and leptin. *Physiol Behav* 99(1):125-130.
176. Quennell JH*, et al.* (2009) Leptin indirectly regulates gonadotropin-releasing hormone neuronal function. *Endocrinology* 150(6):2805-2812.
177. Smith JT, Acohido BV, Clifton DK, & Steiner RA (2006) KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *J Neuroendocrinol* 18(4):298-303.
178. Sainsbury A*, et al.* (2002) Y4 receptor knockout rescues fertility in ob/ob mice. *Genes Dev* 16(9):1077-1088.
179. Hummel KP, Dickie MM, & Coleman DL (1966) Diabetes, a new mutation in the mouse. *Science* 153(3740):1127-1128.
180. Lamia KA, Storch KF, & Weitz CJ (2008) Physiological significance of a peripheral tissue circadian clock. *Proc Natl Acad Sci U S A* 105(39):15172-15177.
181. Turek FW*, et al.* (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 308(5724):1043-1045.
182. Ho A & Chin A (1988) Circadian feeding and drinking patterns of genetically obese mice fed solid chow diet. *Physiol Behav* 43(5):651-656.
183. Horvath TL & Wikler KC (1999) Aromatase in developing sensory systems of the rat brain. *J Neuroendocrinol* 11(2):77-84.
184. Fisher CR, Graves KH, Parlow AF, & Simpson ER (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci U S A* 95(12):6965-6970.
185. Eskes GA (1984) Neural control of the daily rhythm of sexual behavior in the male golden hamster. *Brain Res* 293(1):127-141.
186. vom Saal FS (1989) Sexual differentiation in litter-bearing mammals: influence of sex of adjacent fetuses in utero. *J Anim Sci* 67(7):1824-1840.

187. Stocker CJ, Arch JR, & Cawthorne MA (2005) Fetal origins of insulin resistance and obesity. *Proc Nutr Soc* 64(2):143-151.
188. Hamada T, Honma S, & Honma K (2011) Light responsiveness of clock genes, Per1 and Per2, in the olfactory bulb of mice. *Biochem Biophys Res Commun* 409(4):727-731.
189. Abe M*, et al.* (2002) Circadian rhythms in isolated brain regions. *J Neurosci* 22(1):350-356.
190. McClelland RJ & Cowley JJ (1972) The effects of lesions of the olfactory bulbs on the growth and behavior of mice. *Physiol Behav* 9(3):319-324.

FIGURELEGENDS

Figure 1-1. Circadian rhythms are biological rhythms with a period of approximately 24 hours. A. double-plotted actograms of mouse activity. Black mark represents wheel running activity in the mouse cage that is monitored by a computer. The bar on top shows the light cycle, with white bars representing the "lights on" period and the black bars representing the "lights off" period. The y axis is the number of days the mouse is monitored. B. gene expression in liver. The x axis is the time points of day and night. The y axis is the gene expression (log_{10}Intensity) .

Figure 1-2. The molecular model of circadian rhythm

Figure 1-3. The general physiology circadian model

Figure 1-4. The circadian control of reproductive axis in females

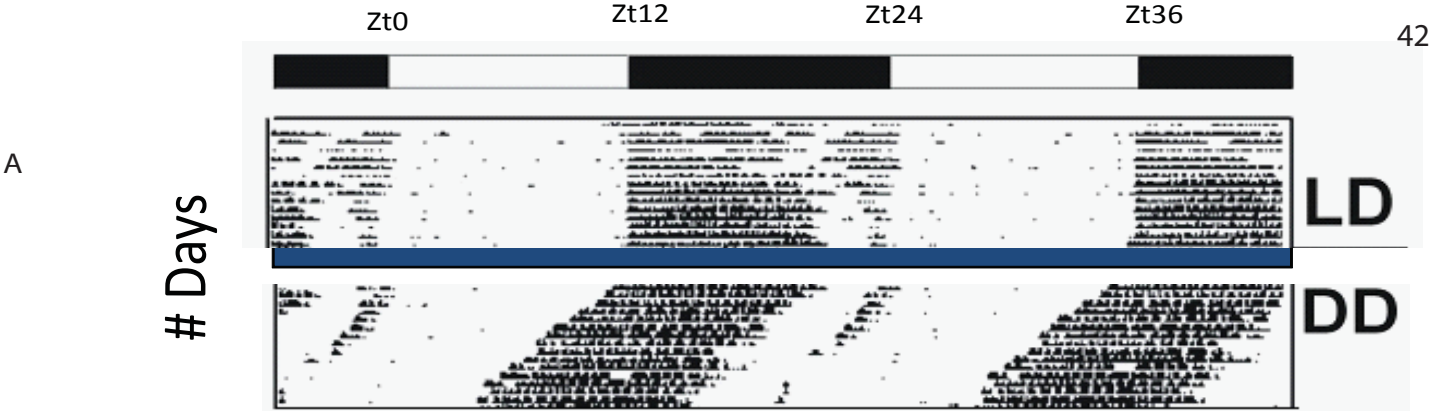
Figure 1-5. The circadian control of reproductive axis in males.

Figure 1-6. The mouse brain and the SCN nucleus

Figure 1-7. The hypothalamus

Figure 1-8. The efferents from hypothalamus to the other part of the brain

Figure 1-9. Summary of synaptic interaction between chemically characterized pre- and postsynaptic neurons. Axons are represented by straight lines, and postsynaptic cells and dendrites by circles. In some cases the identity of the postsynaptic cells is shown; in others two chemically identified axons terminate on an unidentified cell. GRP, gastrin-releasing peptide; AVP, vasopressin; VIP, vasoactive intestinal polypeptide; SRIF, somatostatin; GABA, gama aminobutyrate; 5HT, serotonin. (Adapted from Van den pol and Decavel, 1990).



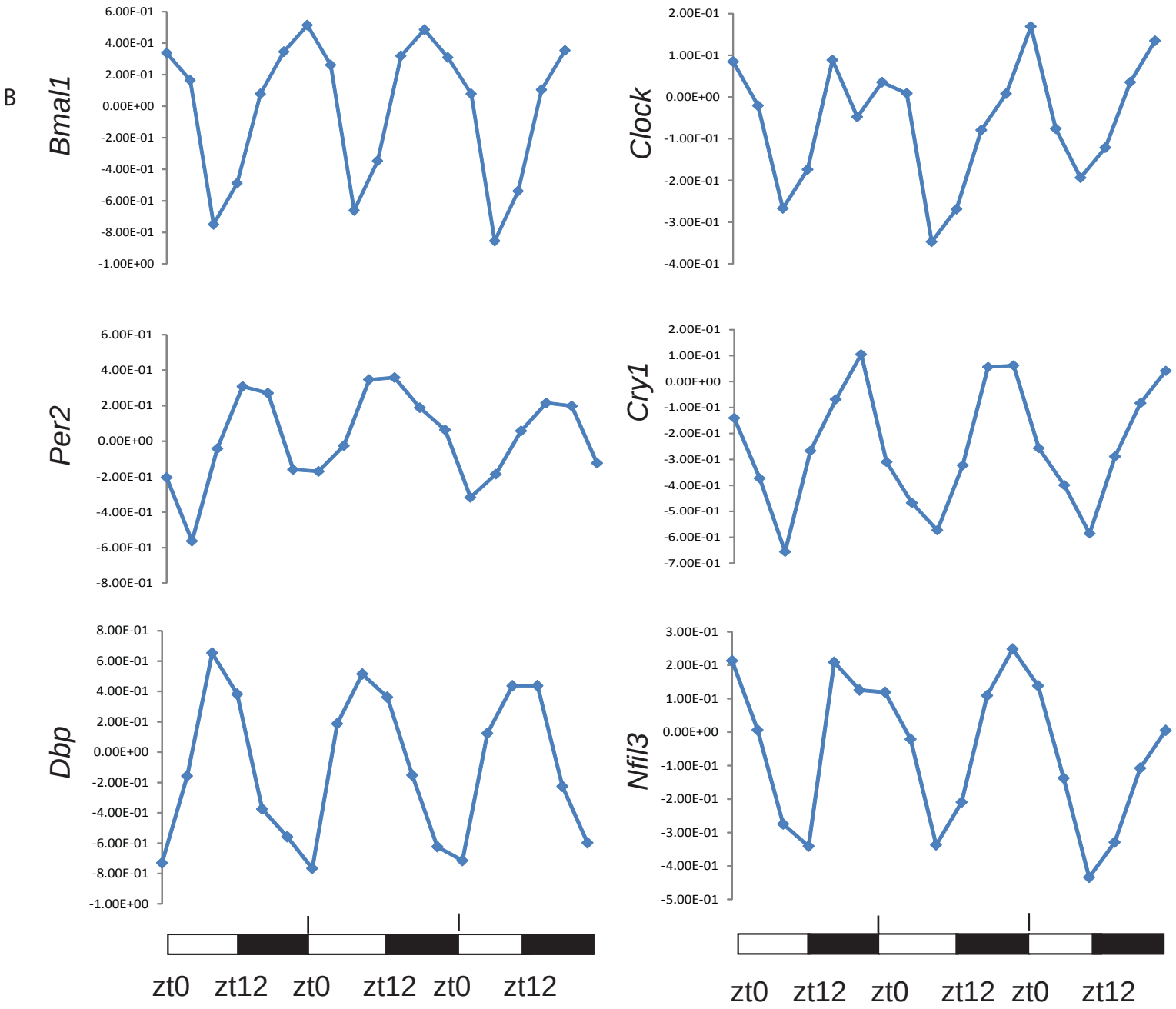
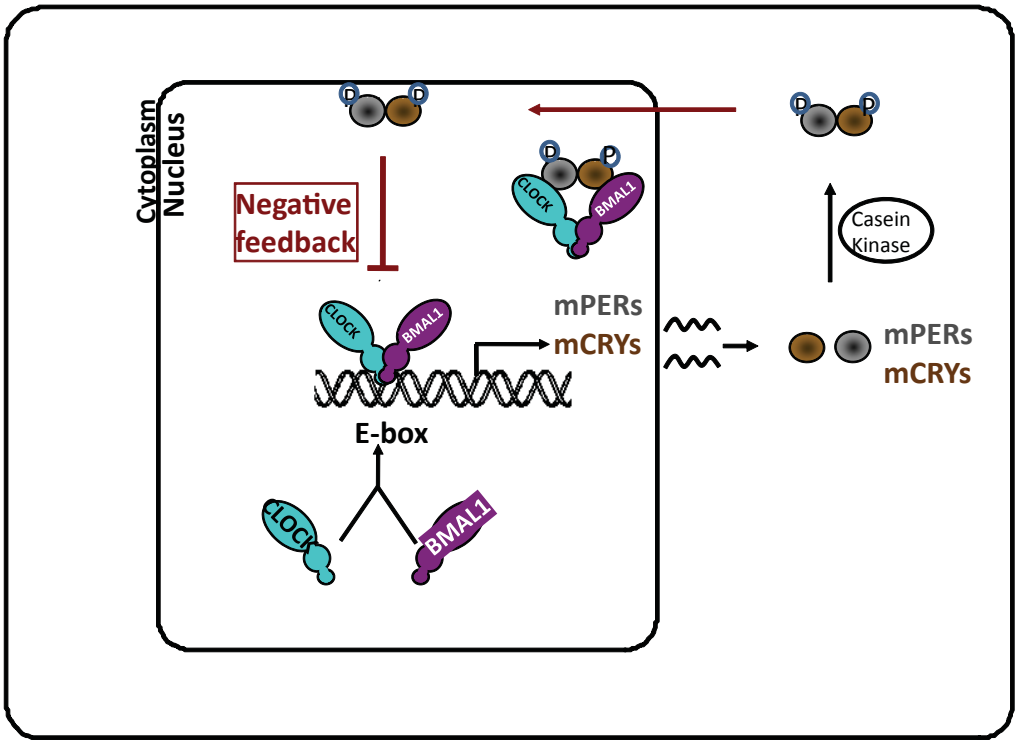
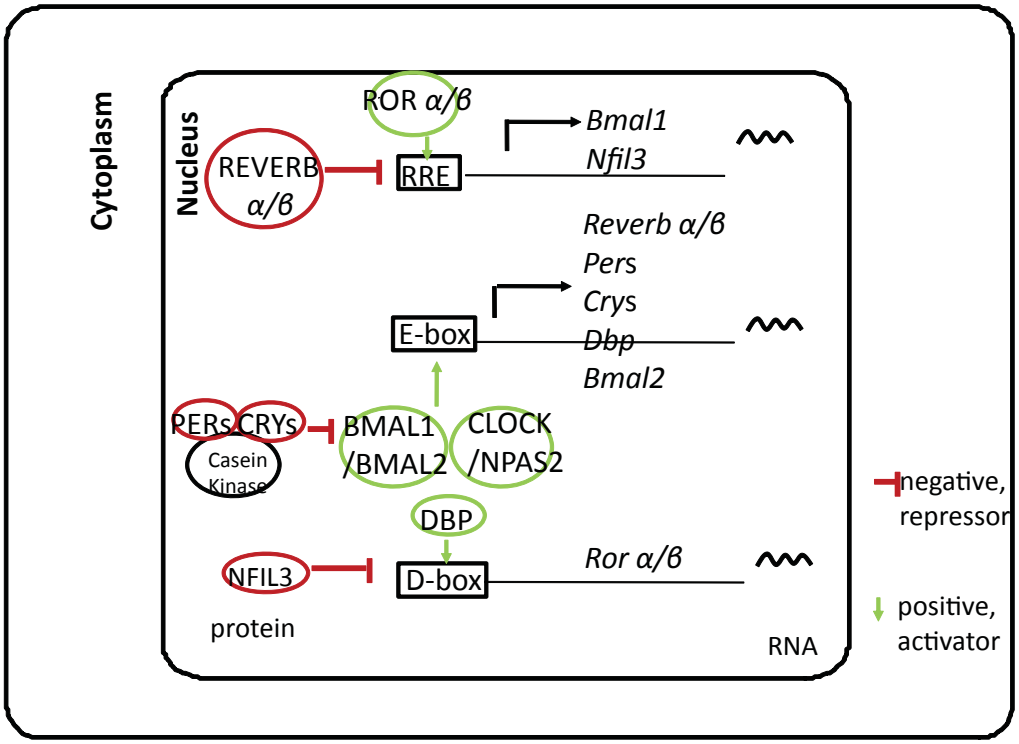


Figure 1-1



A



B

Figure 1-2

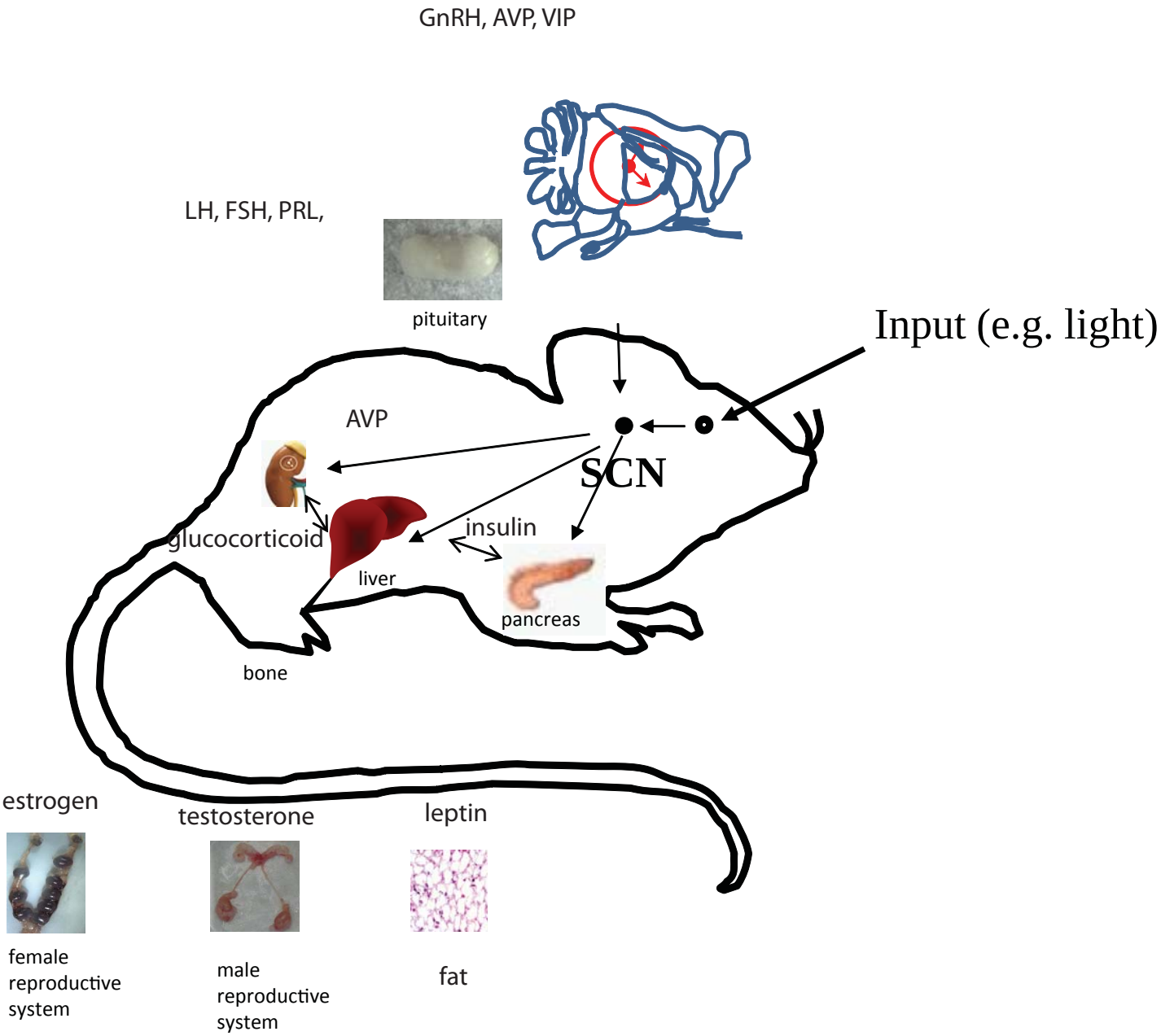


Figure 1-3

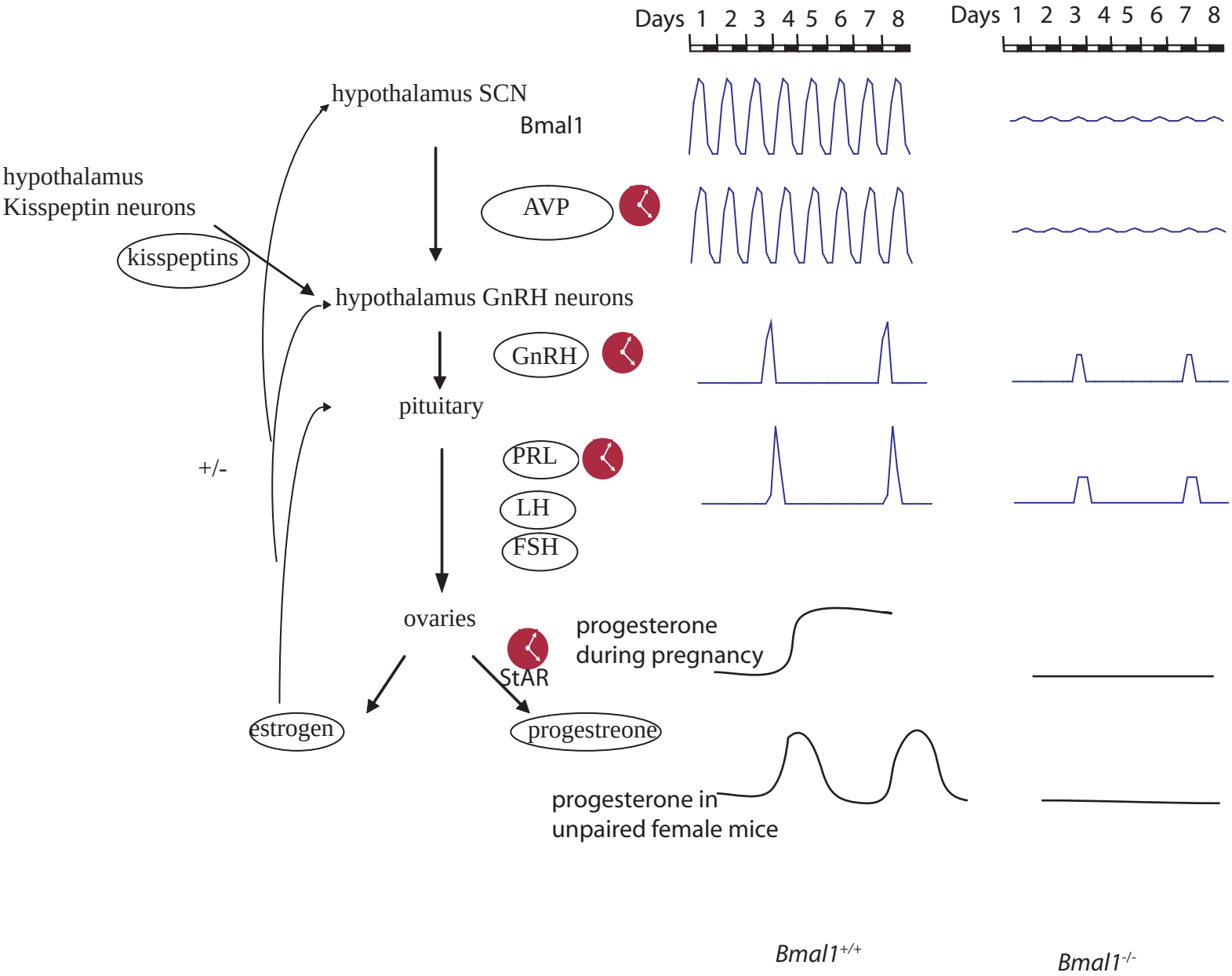


Figure 1-4

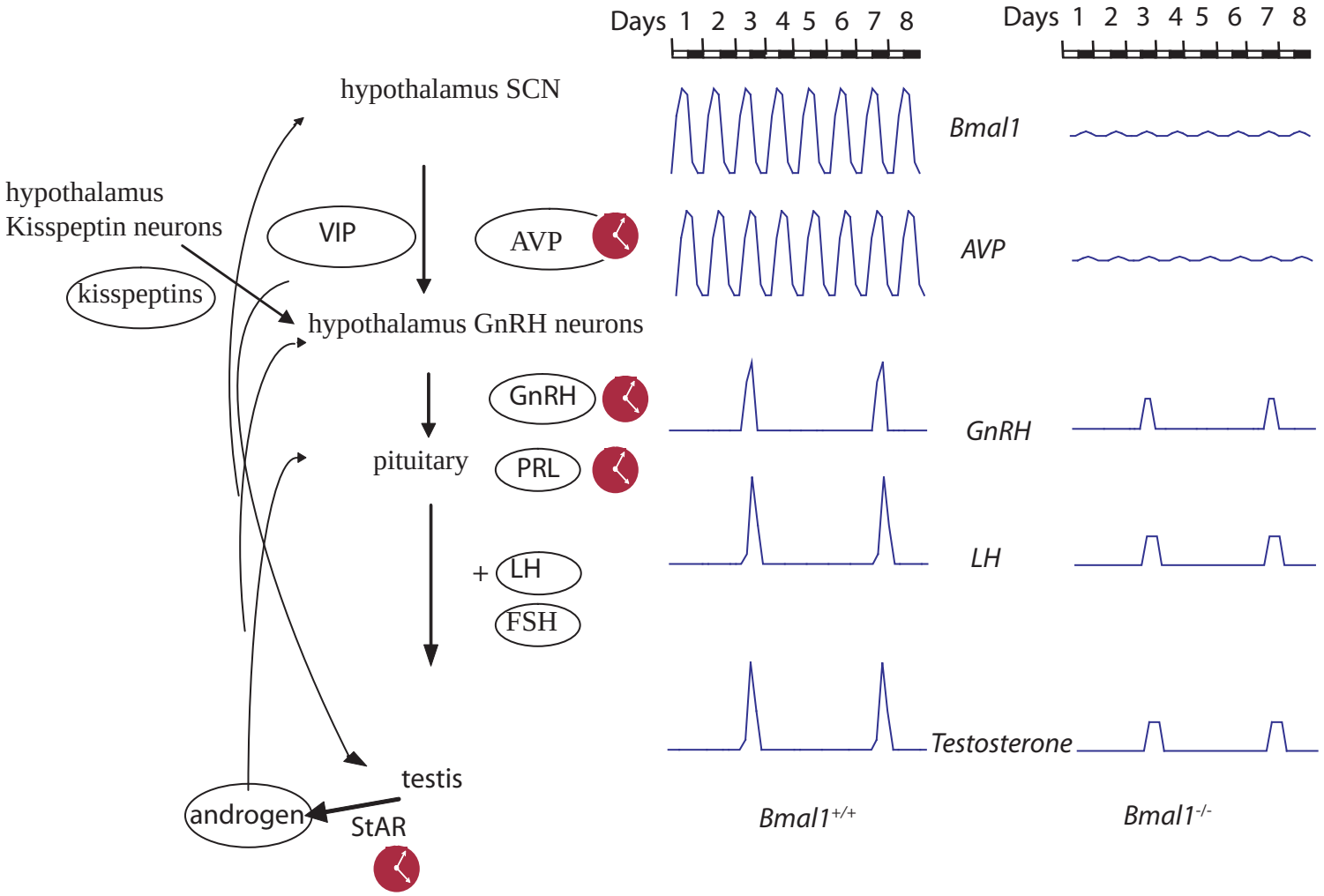
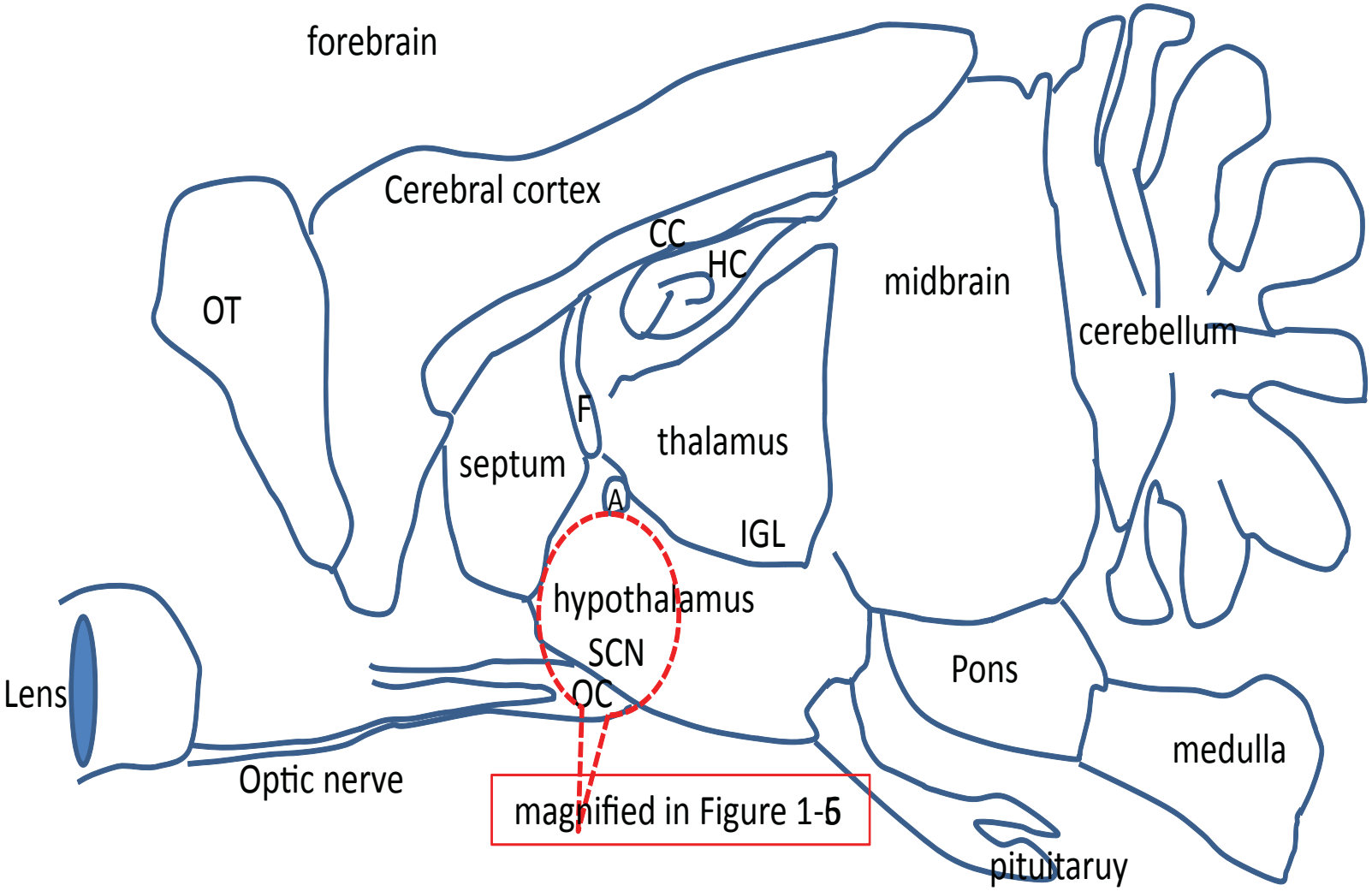


Figure 1-5



OT olfactory tube
CC corpus callosum
F fornix
HC Hippocampus
OC optic chiasma
SCN suprachiasmatic nucleus
IGL intergenicular nucleus
A anterior commissure

Figure 1-6

AR	arcuate nucleus
DMH	dorsomedial nucleus
OC	optic chiasm
POA	preoptic area
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
vSPZ	ventral subparaventricular zone
VMH	ventromedial nucleus
SO	supraoptic area

RHT	retinohypothalamic tract
GHT	geniculohypothathalamic tract
IGL	intergeniculate leaflet

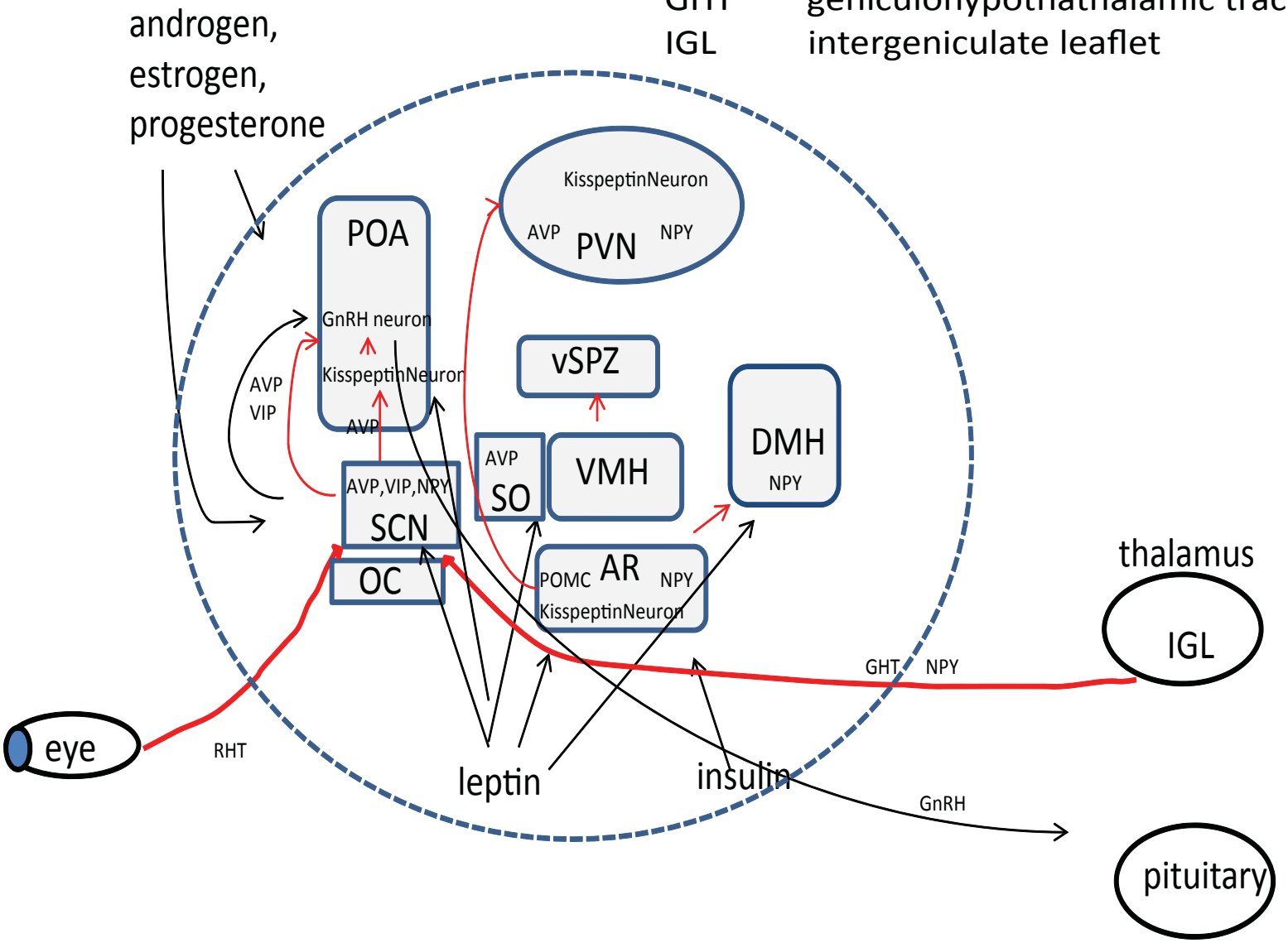


Figure 1-7

Black arrow	hormonal signals
Red arrow	neuronal connections
Sagittal	

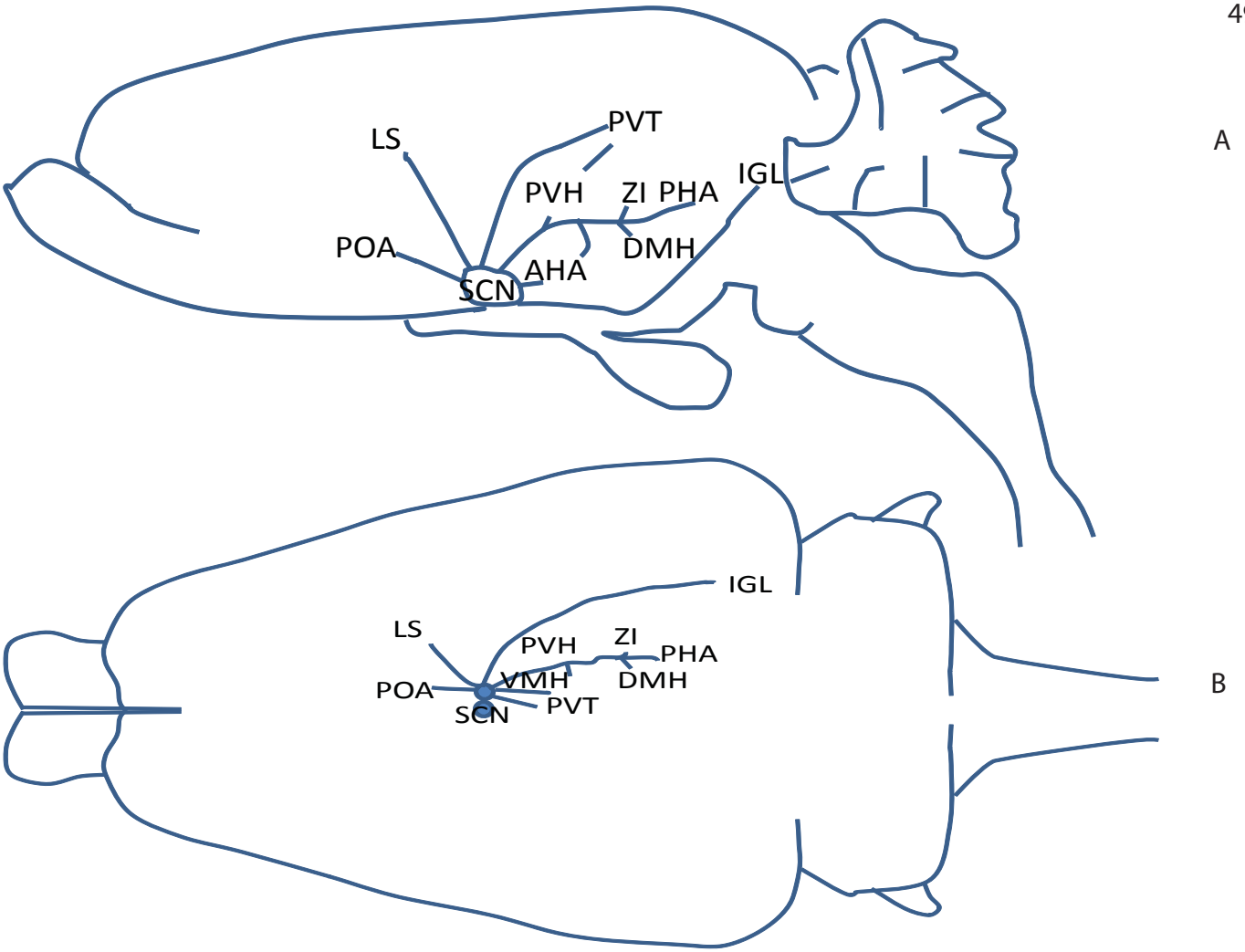


Figure 1-8

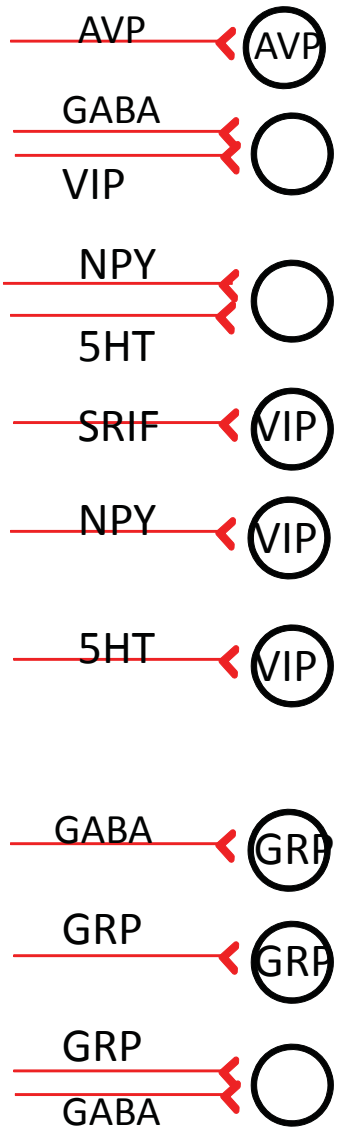


Figure 1-9 (Adapted from Van den pol and Decavel, 1990)

Table 1-1. Summary of the reproductive phenotype of rodent mutant models mentioned in chapter 1.

	Male	Female
Hpg (GnRH mutation)	infertile	infertile
Kiss1$^{-/-}$	Infertile, hypogonadism	Infertile, hypogonadism
GPR54$^{-/-}$	infertile	infertile
Kisspeptin neuron ablation		fertile
Gpr54 neuron ablation		fertile
Brattleboro rats (*Avp* mutation)	subfertility	subfertility
Vip$^{-/-}$	Reduced testosterone, FSH	
Vipr2$^{-/-}$		
Ghr$^{-/-}$	subfertility	subfertility
Prl$^{-/-}$	fertile	infertile, implantation reinitiated by P4
Prlr$^{-/-}$	delay in fertility	Infertile, implantation failure, irregular estrous cycle, impaired mammary glanddevelopment, reduced fertilization rate of oocytes, defective preimplantation embryonic development,

		implantation rescued by P4
Snell dwarf (pit-1 mutation)	Infertile,GH,PRL,TSH deficiency, low gonadotropin and testosterone, long life span, fertility restored by PRL, or GH/thyroxin	infertile,GH,PRL,TSH deficiency, low gonadotropin and testosterone, long life span, fertility restored by PRL
Ames dwarf (prop1 mutation)	Infertile, GH,PRL,TSH deficiency fertility restored by PRL	Infertile, GH,PRL,TSH deficiency, fertility restored by PRL
Cdk4$^{-/-}$		Infertile, hypopituitarism, P4, PRL rescue implantation
IGF1$^{-/-}$	infertile	infertile
ob/ob (lep mutation)	infertile, obese, diabetes, disrupted feeding rhythm	infertile, obese, diabetes, disrupted feeding rhythm
db/db (*lepr* mutation)	infertile, Obese,	infertile, Obese,
Lepr$^{fx/fx}$*Cre*$^{Nkx2.1}$	infertile, obese	infertile, obese
LepR$^{fx/fx}$*Cre*$^{sf\text{-}1}$	fertile	fertile
Lepr$^{fx/fx}$*Cre*^{CamkII}	71.4%fertile	80% fertile (Julie E. McMinn)
lepr$^{fx/fx}$*Cre*^{CamkII}	Subfertile (28% fertile with only 1 litter in 110 days of breeding time, late onset of puberty)	subfertile (Janette H. Quennell)
LepRb$^{fx/fx}$*Cre*^{GnRH}	fertile	fertile (Janette H. Quennell)
Stat3$^{fx/fx}$*Cre*^{lepRb}	fertile, obese	fertile, obese

Stat3$^{fx/fx}$*Cre*^{nestin}	infertile	infertile
Androgen receptor$^{fx/fx}$*Cre*^{Amh}	Infertile, libido normal but azoospermia, defective spermatogenesis	
Androgen receptor$^{fx/fx}$ *Cre*^{Amhr2}	Infertile, libido normal but azoospermia, defective spermatogenesis	
Aromatase$^{-/-}$	50% infertile low libido, obese	Infertile, no corpus luteum, anovulation, obese
StAR$^{-/-}$	female external genitalia, low testosterone, mature spermatids in epididymis	infertile, low p4, normal estrogen, impaired ovulation
Esr1$^{-/-}$	Reduced mounting behavior	infertile, no corpus luteum
Magel2$^{-/-}$ (circadianlly expressed in SCN)	mating rate declined after 16 weeks old finally cease, GH deficiency, reduced orexin expression in hypothalamus neurons,obese, blunted circadian rhythm, reduced activity level	fertile, GH deficiency, reduced orexin expression in hypothalamus neurons,obese, blunted circadian rhythm, reduced activity level
Sf-1$^{-/-}$	infertile, hypoplastic testis, low LH, FSH	infertile, hypoplastic ovaries, low LH, FSH
Sf-1$^{fx/fx}$*Cre*^{Amhr2}	infertile	infertile (no corpus luteum)
Sf-1$^{fx/fx}$*Cre*^{nestin}	fertile	subfertile

CHAPTER II

Targeted disruption of *bmal1* in the peripheral reproductive axis results in infertility in female mice

ABSTRACT

Bmal1 is an essential component of the circadian clock, which plays important roles in various aspects of reproductive biology. Mice with global deletion of *Bmal1* display infertility, irregular estrous cycles in females, and early aging, all of which complicate efforts to delineate the primary versus the secondary effects of *Bmal1* deficiency on fertility. In an effort to determine the roles of peripheral clocks in the reproductive hormonal axis in female fertility, we used Cre/loxP recombination technology to genetically delete *Bmal1* specifically in steroidogenic factor 1 (sf-1) expressing cells (sf-1 expresses in all levels of reproductive axis including hypothalamus, pituitary and gonads). We show that $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females are infertile with implantation failure and loss of visible implantation sites. Moreover, $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females display lower progesterone levels than control females, and progesterone supplementation rescues the numbers of implantation sites back to control levels. Isolation of the clocks of ovaries is supported by the additional observation that transplantation of wild type ovaries into $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females results in 100% fertility. Our study suggests that ovarian *Bmal1* is an essential peripheral clock governing implantation and fertility in female mice.

INTRODUCTION

In mammals, circadian physiology is controlled by a molecular clock that is present in virtually every cell type. At the core of this molecular clock is the CLOCK: BMAL1 (also known as MOP3 and ARNTL) dimer that transcriptionally activates feed-back components, such as the *Per*1-3, *Cry*1-2, Rorα, and the *Nr1d1* genes (1). Additional "output" genes important in regulating circadian physiology include *Dbp, Avp, and StAR* (2, 3). The core clocks within cells of peripheral tissues appear to be synchronized by a master clock within the suprachiasmatic nucleus (SCN) of the hypothalamus (4).

The importance of circadian rhythms in reproductive biology is demonstrated by epidemiological evidence indicating that "shift work" in women is associated with menstrual irregularities and reproductive difficulties (5). Genetic studies from animal models support the relationship between circadian physiology and fertility. More specifically, mutations in components of the core circadian clock have deleterious effects on reproductive outcomes. For example, global *Bmal1*$^{-/-}$ females are infertile and *Clock*$^{\Delta 19}$/*Clock*$^{\Delta 19}$ mice display irregular estrous cycles, absence of luteinizing hormone (LH) surges and higher rates of fetal reabsorption (6). Finally, as *Per1*$^{-/-}$ and *Per2*$^{-/-}$ mice age, they bear smaller litters as compared to their wild type controls (7).

Although the relationship between circadian clocks and reproduction is clear, the mechanisms underlying this relationship and the relative contributions of the master and peripheral clocks remain undefined. Evidence for the importance of the master clock in reproduction comes from a number of observations. For example, the SCN regulates secretion of hormones essential for reproduction such as gonadotropin-releasing-hormone (GnRH) and

kisspeptin via direct projections and secreted mediators (e.g., AVP). Moreover, lesioning of the SCN in female rats leads to irregular estrous cycles and persistent vaginal cornification (8, 9). Evidence for the importance of peripheral clocks is less clear and is supported by the observation of oscillating expression of core clock genes and circadian output genes in the major tissues of the reproductive axis (e.g., GnRH neurons, anterior pituitary, and steroidogenic cells in the gonads) (10).

In an effort to delineate the role of the peripheral clocks in female reproductive physiology, we have chosen to use conditional genetic ablation of the core clock gene, *Bmal1*. To date, global *Bmal1*$^{-/-}$ is the only single locus deletion in mice that provides significant loss of core circadian functions (11). Prior studies of global *Bmal1*$^{-/-}$ mice support the idea that this core clock gene plays a significant role in multiple aspects of reproductive biology. In this regard, global *Bmal1*$^{-/-}$ females display delayed vaginal opening, reduced ovarian weight, irregular estrous cycles, implantation failure, and reduction in ductal length and branches of the mammary gland (12, 13). Our preference for the use of conditional null alleles comes from the observation that the use of global null alleles of core clock or output genes does not allow distinction between relative roles of the master vs peripheral clocks. Some of the uncertainties from the use of the global null alleles arise because the central clock can alter the functions of the peripheral clocks through synchronizing signals such as food intake, leptin levels, and insulin levels. (14-17).

Conditional knockout mouse models have great potential to help us understand the relative roles of the master clock and peripheral clocks in reproduction and also allow us to segregate other significant circadian phenotypes from this biology. In this regard, global *Bmal1*$^{-}$

$^{/-}$ mice display pervasive arthropathy by 20 weeks of age (18, 19), premature early aging (20-22), and diabetes (23-25). Any of these defects could indirectly influence reproductive function (10, 12). Through the use of conditional knockout alleles and ovary transplantation, we are able to study the roles of cell specific circadian clocks in female mice.

MATERIALS AND METHODS

Generation of *Bmal1*^{fxneo} Mice (performed by Jaquecline Wallisser and Susan Moran).

In order to generate conditional *Bmal1*$^{fx/fx}$ mice, we first generated the *Bmal1*^{fxneo} line through homologous recombination in embryonic stem cells. Generation of the *Bmal1*^{fxneo} targeted allele involved amplifying 7.8 kb and 2.0 kb regions of homology to the *Bmal1* genomic DNA isolated from BAC DNA (Genome Systems, St. Louis, MO). The 2.0 kb short arm homology region was cloned into a targeting vector (PL1169), which contains the neomycin resistance gene flanked by Frt sites for later excision by *Flp* recombinase. To generate PL2025, a 0.6 kb region encompassing exon 4, the region encoding the basic-helix-loop-helix domain, was amplified from *Bmal1* genomic DNA and a loxP site was added to the 3' end in the opposite orientation. The exon 4 region was cloned into to the *Sal*I site of PL2025 to generate PL2027. This cloning step produced a floxed bHLH domain since the targeting vector contained an internal loxP site. Finally, the 7.8 kb long arm of homology was cloned into the *Sac*II site of the targeting vector to produce the final *Bmal1*^{fxneo} targeting construct (PL2029) (Fig.2-1A). Correct cloning and orientation of the various molecular components of the targeting construct was verified at each step by sequencing and diagnostic restriction digests.

Homologous recombination in GS1 embryonic stem cell 129 strain (Genome Systems) was performed. Embryonic stem cell clones were screened for homologous recombination by Southern blot of *EcoR*I digested genomic DNA using a 180 bp probe amplified from PL1225 using the OL2281/2283 primer pair. This probe is located just upstream of the 5'end of the targeting construct. Correctly targeted clones were injected into 3.5-day postcoital C57BL/6J blastocysts and resulting chimeras were backcrossed to C57BL/6J mice to determine germ line transmission of the targeted allele and were verified by Southern blot of tail biopsies (Fig. 2-1B).

Animals transmitting the *Bmal1*^{fxneo} mutation, which included the exon 4 region flanked by loxP sites and the neomycin resistance gene flanked by Frt sites, were backcrossed to C57BL/6J mice. All oligo sequences are shown in table 2-1.

Generation of Conditional *Bmal1*^{fx} Mice (performed by Jaquecline Wallisser and Susan Moran).

The conditional *Bmal1* allele (*Bmal1*^{fx}) contains exon 4 of *mBmal1* flanked by loxP sites ("floxed", fx). The *Bmal1*^{fx} mice were generated from *Bmal1*^{fxneo} allele by crossing to a transgenic line carrying *Flp* under the control of the Rosa promoter (*Flp*^{Rosa}, strain name 129S4/SvJaeSor-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J) (Jackson Laboratories, Bar Harbor, ME) (Fig. 2-1A). Progeny testing positive for Flp were further screened for excision of the neomycin cassette through *Flp* recombination, leaving a single Frt site remaining. Identification of the neomycin-excised allele was accomplished through PCR amplification of the region surrounding the remaining Frt site in the excised allele using OL5439/5440 (Fig.2-1C). Following confirmation of neomycin excision, mice carrying the floxed exon 4 and the *Flp* transgene (*Bmal1*$^{fx/+}$*Flp*^{Rosa}) were then backcrossed to C57BL/6J to remove the *Flp*^{Rosa} transgene and produce the parental line (*Bmal1*$^{fx/+}$). Mice heterozygous for the *Bmal1*$^{fx/+}$ allele were backcrossed to C57BL/6J for 10 generations and then intercrossed to produce homozygous conditional *Bmal1*$^{fx/fx}$ mice. Routine genotyping of the conditional allele was accomplished with OL6013/5436, which detected the presence of the intact *lox*p site immediately downstream of exon 4 (Fig.2-1C). Partial maps of the structural *Bmal1* gene, the *Bmal1*^{fxneo} allele and the conditional *Bmal1*^{fx} allele are shown (Fig.2-1A). A PCR-based protocol was developed to genotype *Bmal1*^{fx} progeny based on detection of the *lox*P sequence upstream of exon 4 (Fig.2-1D). To confirm that expression from the *Bmal1*$^{fx/fx}$ allele was similar to that of the wild-type allele, we performed a northern blot

analysis on 3µg polyA RNA isolated from whole brain using the RNeasy Lipid Kit (Qiagen, Valencia, CA) (Fig.2-1E). The probe used to detect *Bmal1* was a 600 bp fragment generated from a KpnI digest of a *Bmal1* image clone (clone number 3980719, Open Biosystems, Huntsville, AL). The primer pair OL6013/5436 amplifies the intact conditional alleles (~360 bp). The *Bmal1*^{fx}-excised allele is amplified with the OL6013/6014 primer pair and generates a ~450 bp band due to the loss of the floxed region and residual *lox P* and *Frt* sites remaining after excision.

Gene targeting and animal husbandry.

To disrupt the circadian clock in the reproductive axis, mice harboring the *Bmal1*^{fx} allele were bred to mice expressing a *Cre* transgene driven by the s*f-1* (also known as *Nr5a1*) promoter (26). The Sf-1/cre mouse line (from Keith L Parker, University of Texas Southwestern) were breeding to C57BL/6J background for 7 generations. Sf-1/cre mice are outwardly healthy and breed well. Sf-1/cre conveys recombination in the somatic cells of the gonads (ovarian theca and granulosa cells), the adrenal cortex, the anterior pituitary (gonadotrophs), the spleen, and the ventromedial hypothalamic nucleus as early as embryonic day 10.5.

Sf-1/cre mice were crossed with the conditional *Bmal1*$^{fx/fx}$ mice. Breedings were set up so that only one copy of *Cre* transgene existed in the offspring (hemizygous, denoted Cre^{sf-1}). Mice homozygous for the floxed allele and hemizygous for Cre were used as experiment groups. Littermates that were negative for the *Cre* transgene (*Bmal1*$^{fx/fx}$ or *Bmal1*$^{fx/+}$) or positive for the C*re* transgene while encompassing a wild type allele (*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$) were used as control groups.

Genotyping.

For genotyping, genomic DNA from tail and ear clips were isolated using "PUREGENE DNA Isolation kit" (Gentra Systems, Inc., Minneapolis). Genotyping for the *Cre* transgene was performed by PCR using the forward primer, OL2642 and reverse primer, OL2643 in a reaction consisting of 2.5 units *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM MgCl2, 1% Triton X-100, 200 µM dNTPs and 0.1 µM of each primer. The PCR reaction was carried out using the following conditions : (95°/5 min→[95°C/30 s→ 60°C /30 s→ 72°C /30 s]x 30cycles→72°/5 min → 4°/∞). A 450 bp band confirmed the presence of the *Cre* transgene. Samples negative for *Cre* did not amplify a product.

Genotyping for *Bmal1* was using a combination of the following PCR primers (positions of the oligos on the alleles are shown in Fig. 2-1), OL5436, OL6013 and OL6014, in a PCR program with the conditions (95°/5 min → [95°/30 s → 61°/30 s → 72°/5 s] x 31cycles → 72°/5 min → 4°/∞). The wild-type allele (wt or +) generates a ~310 bp band while the floxed allele (fx) generates a ~360 bp band. The excised allele (ex) is amplified with the OL6013/OL6014 primer pair and generates a ~450 bp band.

All procedures were approved by the Animal Care and Use Committee, University of Wisconsin. Mice were housed under normal 12hr: 12hr light: dark cycle with standard lab chow (Mouse diet 9F 5020) (PMI Nutrition International, LLC, Brentwood, MO 63144) and water ad libitum.

Assessment of feeding rhythms.

Each mouse was housed singly for a week before the assessment of feeding rhythms. To this end, a known amount of food was provided on day 1 and food weight was measured at Zeitgeber Time 0 (ZT0, lights on) and ZT12 (lights off) for 10 consecutive days. For each 12-hour period, the difference in food weight was taken as a measure of food consumption for that

period. Measurements were carried out in the same room over the same time period to control for changes in food weight due to humidity.

Assessment of estrous cycle.

Estrous cycles were examined in females from 10 weeks of age. Vaginal smears were taken at ZT4 for at least 22 consecutive days. Vaginal secretions were collected with a plastic pipette filled with 10 µL PBS by inserting the tip just into the vaginal opening. Cell morphology was analyzed under a microscope to determine cycle stage (27). The characterization of each phase is based on the proportion of the three types of cells observed in the vaginal smear: nucleated round cells (proestrus), cornified cells (estrus) and leukocytes (diestrus).

Histological analysis.

For histological characterization, tissues were fixed in 10% buffered formalin overnight, dehydrated in graded ethanol and embedded in paraffin. Sections (7µm) were stained with hematoxylin and eosin (H&E) and analyzed under light microscopy as described (28) . For some experiments, 100 µl 1% (w/v) Chicago sky blue (Sigma, St. Louis, MO) was injected through the tail vein as indicated 5 minutes prior to euthanasia. The female reproductive tracts were then dissected and sectioned for histological study as described (29). For the corpora lutea counting experiment, serial sections were cut on the $Bmal1^{fx/fx}Cre^{Sf\text{-}1}$ ovaries on 3.5 dpc, and the single section with the biggest number of coropa lutea was taken as the representative section for that ovary.

Hormone measurements.

Mice were euthanized with carbon dioxide gas inhalation in their home cages. Whole blood samples were collected from the heart. Serum was separated by centrifugation and stored

at -30°C until assayed. Progesterone and prolactin levels were analyzed by Radioimmunoassay in the laboratory of National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, California).

In Vitro fertilization and embryo handling (performed by Kathy J. Krentz).

In vitro fertilization (IVF) procedures and other embryo handling were followed standard protocols (29). Briefly, global *Bmal1*$^{-/-}$ *or Bmal1*$^{+/+}$ female mice between 23-32 days of age were treated by intraperitoneal injection with 5 IU pregnant mare serum gonadotropin (PMSG) (Sigma, St. Louis, MO) at ZT15 followed by 2.5 IU human chorionic gonadotropin (hCG) (Sigma, St. Louis, MO) 48 hours later. Thirteen hours after HCG administration, females were euthanized and cumulus-intact eggs released from the oviducts. Sperm was obtained fresh from the cauda epididymis from global *Bmal1*$^{-/-}$ *or Bmal1*$^{+/+}$ mice. After co-incubation of the eggs and sperm, two-cell embryos were transferred to outbred ICR pseudopregnant females.

Ovary transplantation(performed by Kathy J. Krentz).

Ovary transplantation was carried out using female mice between the age of 80 and 110 days (29). Briefly, mice were anesthetized by inhalation through a nose cone of 2% isoflurane (Halocarbon Products Corp, River Edge, NJ, USA) mixed with oxygen using a V7276 anesthesia machine (Surgivet Veterinary Surgical Products, Norwell, MA). The mice received a subcutaneous injection of buprenophine (0.05-0.1 mg/kg, Sigma, St. Louis, MO) and were placed on a heated disinfected pad. Donor ovaries were surgically removed through a tiny incision of the bursa and inserted into the bursa of the recipient under a dissecting microscope. Both (bilateral) ovaries were exchanged between 12 *Bmal1*$^{fx/fx}$ and 12 *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$. Transplantation was unsuccessful with one recipient *Bmal1*$^{fx/fx}$ female and this female is

excluded from our results. All 23 females recovered normal movement one hour after the surgery. Females were mated to wild type proven fertile males 21 days after the surgery.

Hormone supplementation.

To try to rescue the infertility in *Bmal1*$^{-/-}$ or *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females, we treated females with two different hormone supplementation protocols. First, starting on 3.5 dpc, *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females were injected daily with 2 mg progesterone (Sigma, St. Louis, MO) dissolved in corn oil subcutaneously (s.c.) at ZT4. 100 µl Corn oil (Sigma, St. Louis, MO) used as vehicle control (13). Second, starting on 1.5 dpc *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females were injected 150 µg prolactin (Sigma, St. Louis, MO) dissolved in saline twice daily at ZT0 and ZT12, or 100 µl saline as vehicle control (30). On 6.5 dpc or 10.5 dpc mice were euthanized to check for implantation sites.

RNA Isolation.

At ZT12, 3.5 dpc females were euthanized, and uteri were flushed to confirm the presence of embryos. Ovaries were collected from pregnant females. RNA was isolated using the QIAGEN RNeasy kit (Qiagen, Valencia, CA). RNA quality and concentration were assessed using Agilent bioanalyzer2100 (Agilent, Technologies, Santa Clara, CA) and a NanoDrop spectrophotometer (Thermo scientific, Wilmington, DE).

Microarray Analysis

Microarray analysis of RNA expression was carried out using the Agilent two-color microarray-based gene expression analysis protocol and whole mouse genome microarray (G4122F, Agilent Technologies, Santa Clara, CA), which represents 39430 mouse transcripts. For each array analysis, a pool of 3 RNA samples from 3 individual *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ ovaries labeled with cy3 were cohybridized against a pool of 3 RNA samples from *Bmal1*$^{fx/fx}$ ovaries labeled with cy5. A total of 3 microarrays were hybridized resulting in 9 RNA samples for each

genotype. Microarray images were quantified and data was extracted by Agilent feature extraction software 9.2 (Agilent Technologies, Santa Clara, CA). The hybridization characteristics were verified by the internal control probe sets (spike-in kit, cat No.5188-5279, Agilent Technologies, Santa Clara, CA) using feature extraction quality control metrics (31).

Statistical Analysis

The Wilcoxon rank sum test (two-sided) was used in all hypotheses testing for difference in locations. The Chi-square test was consulted in all hypotheses testing for difference in proportion (Statistical problems in genetics and molecular biology, Norman Drinkwater and Carter Denniston).

RESULTS

Conditional *Bmal1* knockout mice enable evaluation of role of peripheral clocks in fertility.

Bmal1$^{fx/fx}$ mice are outwardly normal and breed normally. To examine the specificity of excision mediated by Cre^{sf-1}, we analyzed various tissues for the presence of the *Bmal1*^{fx}-excised and -unexcised alleles. As shown in Fig 2-1F, in *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females the *Bmal1*^{fx} is excised in the brain, pituitary, adrenal gland, and ovaries but not in liver, muscle, uterus and oviduct. That is consistent with previous reports that Cre^{sf-1} could be used to drive specific deletions in the reproductive axis. In order to characterize the effect of excision of *Bmal1*^{fx} with sf-1/cre on circadian locomotive patterns, we first determined the feeding rhythms of *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice. Wild type C57BL/6J mice have a circadian feeding rhythm with more food intake at night than in the day (32). As shown in Fig. 2-2A, wild type control females display normal circadian feeding rhythms, consuming 3.3 times more food at subjective night than in the subjective day ($P<0.001$). In contrast, global *Bmal1*$^{-/-}$ females displayed a mean night to day ratio of only 1.3, consistent with a disrupted SCN master clock. The *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females exhibited robust circadian feeding rhythm, with night to day ratio of 3.5, suggesting a normal functioning SCN master clock.

As early as 12 weeks of age, global *Bmal1*$^{-/-}$ mice develop an arthropathic phenotype with 100% penetrance (18). In particular, these mice become stiff and have difficulty righting themselves. In addition, body weight is significantly reduced from control values by 20 weeks of age. These arthropathic phenotypes do not occur in *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ mice, as *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice move freely and flexibly without stiffness through the observed life span up to 210 days of

age. Moreover, unlike global *Bmal1*$^{-/-}$ mice, the hindlimbs of 6 month old *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice stained with alizarin red (to detect calcification of ligaments and tendons (18, 33)) exhibited no visible ossification (Fig.2-2B). Unlike the *Bmal1*$^{-/-}$ mice, *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females appeared healthy at 210 days of age with no reduction in body weight, further evidence of the absence of an early aging phenotype (Fig.2-2C). In summary, *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice are outwardly healthy and normal.

***Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females are infertile although they progress through estrous cycles.**

Animals with normal molecular clocks (4 *Bmal1*$^{fx/fx}$ and 1 *Bmal1*$^{fx/+}$*Cre*$^{Sf-1}$) display 1-2 days of diestrus, followed by 1-2 days of proestrus, and then 2-3 days of estrus, resulting in an overall 4-6 day "cycle length" (Fig. 2-3A). *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females (n=6) progressed through estrous cycles with normal cycle length (5.3 ± 0.5days).

To assess fertility, *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females and controls were mated to wild type proven fertile males, starting around 8 weeks of age. Females were scored for ability to produce live pups in a 3-month period. As shown in Table 2-2, none of the *Bmal1*$^{-/-}$ (0/13) or *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ (0/12) females delivered any pups, eventhough, the regular appearance of vaginal plugs every 6-8 days in *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females indicated successful mating. In addition, *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females displayed wide vaginal openings and cornified cells in vaginal smears after 6.5 dpc, characteristic of early entry into estrus and early pregnancy loss. In contrast, 95% of wild type control (either *Bmal1*$^{fx/+}$*Cre*$^{Sf-1}$ or *Bmal1*$^{fx/fx}$) females gave birth to pups. Thus, although *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females progress through the estrous cycle and are capable of mating, they are not able to produce live litters.

***Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females display implantation failure**.

To determine the stage responsible for infertility of *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females, we paired the females with wild type proven males. After pairing, females were examined for presence of copulation plugs early in the day. This was repeated for 14 continuous days or until a plug was found (0.5 dpc). To check for ovulation, uteri were flushed at ZT12 on 3.5 dpc. Implantation was evaluated at ZT12 from 6.5 dpc to 10.5 dpc. Decidual swellings were observed directly (6.5 dpc to 10.5 dpc) or implantation sites were visualized with Chicago sky blue dye (6.5 dpc). As shown in Table 2-3, *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females exhibited normal rates of mating (63/69). Embryos were detected in the uteri of *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ on 3.5 dpc. Histological analysis of the *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ ovaries at 3.5 dpc demonstrated that *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females displayed similar numbers of corpora lutea (4.6 ± 0.5/section in 13 ovaries) compared to the wild type controls (5 ± 0.7/section in 6 ovaries) (Fig. 2-3B). However, most (20 out of 21) *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females did not exhibit normal implantation sites at 6.5 dpc (similar to Fig. 2-5B1). This was significantly different from wild type controls ($P < 0.001$). The remaining *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ one female displayed smaller swellings than wild type females, and no embryos were found on histologic examination.

***Bmal1*$^{-/-}$ embryos survived in wild type pseudopregnant females.**

To evaluate the ability of the *Bmal1*$^{-/-}$ embryo to implant and develop, we used IVF and transferred two-cell stage *Bmal1*$^{-/-}$ embryos into wild type outbred ICR pseudopregnant females. The *Bmal1*$^{-/-}$ females respond to exogenous gonadotropins (PMSG and hCG) and produce viable oocytes. Moreover, these eggs could be fertilized by sperm from *Bmal1*$^{-/-}$ males and develop into two-cell embryos *in vitro*. After transplantation into pseudopregnant females, these embryos developed normally, and survived to weaning (21 days after birth). As shown in table 2-4, the

survival rate of the *Bmal1*$^{-/-}$ embryos was not significantly different from control *Bmal1*$^{+/+}$ embryos.

Bmal1$^{fx/fx}$_Cre_$^{Sf\text{-}1}$ females have decreased levels of progesterone at 3.5 dpc and 6.5 dpc.

Progesterone secretion by the corpus luteum is essential for implantation and remains high throughout gestation regardless of time of day (34). Both global *Bmal1*$^{-/-}$ and *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females had significantly reduced serum progesterone levels compared wild type controls at 3.5 dpc and 6.5 dpc (Fig. 2-4A and 2-4B).

Microarray gene expression shows evidence of impaired ovarian steroid biosynthesis in _Bmal1_$^{fx/fx}$_Cre_$^{Sf\text{-}1}$ females at 3.5 dpc.

In early gestation, the ovaries are the main source of progesterone (35). Therefore we examined global mRNA expression in ovaries of *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females immediately prior to implantation at ZT12 on 3.5 dpc. A cutoff list (intensity > 40, fold change > 1.5, and spot p-value < 0.05, see Agilent G2567AA feature extraction software) and Limma tests ($P < 0.001$, adjust $P < 0.385$, correction mode: false discovery rate) was used to generate a gene list (table 2-5A) used in the David bioinformatics gene ontology analysis (http://david.abcc.ncifcrf.gov/) (36). As shown in table 2-5C, the G-protein coupled receptor protein signaling pathway and the steroid biosynthetic processes were affected. The reduction in mRNA expression of luteinizing hormone receptor (*Lhcgr*) and steroidogenic acute regulatory protein (*StAR*) suggest altered function of the corpus luteum in *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females. In addition, core clock component genes were deregulated (table 2-5B), indicating a disrupted luteal clock.

Transplantation of wild type ovaries to _Bmal1_$^{fx/fx}$_Cre_$^{Sf\text{-}1}$ _females_ rescues infertility.

In order to test whether the infertility of *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females is due to ovarian clock

defects, we exchanged ovaries between *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ and *Bmal1*$^{fx/fx}$ females. As shown in table 2-6, all of the *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ recipients carrying *Bmal1*$^{fx/fx}$ ovaries gave birth to live pups within a 2 month breeding period. In contrast, only 4 out of 11 *Bmal1*$^{fx/fx}$ recipients carrying *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ ovaries gave birth to pups ($P<0.001$). The remaining females displayed evidence of regular mating (6-10 plugs were observed in 6 females during 2 months of observation, 4 plugs in a single female), usually once every 6-10 days, indicating that mating behavior was not affected. These results support the hypothesis that the ovarian clock is a primary determinant of female fertility.

Progesterone supplementation rescued implantation failure in *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females.

We asked if progesterone, one of the key hormones produced by the ovaries, could rescue implantation. As shown in Table 2-7 and Fig. 2-5B, none of the vehicle-treated *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females showed evidence of implantation on 6.5 dpc. In contrast, 5 out of 8 *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females supplemented with progesterone exhibited successful implantation ($P < 0.005$ vs corn oil treatment) (Fig. 2-5C) with similar number of implantation sites compared to controls ($P > 0.05$) (Fig. 2-5A). To test whether progesterone rescued-pregnancies could be sustained beyond 6.5 dpc, we injected 5 females with progesterone until 10.5 dpc. Three displayed histologically normal embryos (Table 2-8, Fig. 2-5G), with numbers of implantation sites and overall embryo development similar to that of wild type controls (Fig. 2-5E).

Prolactin supplementation only transiently rescued implantation failure in *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females.

Since *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females showed lower prolactin levels than wild type controls on 3.5 dpc but not 6.5 dpc (Fig. 2-4C, D), we evaluated the effects of supplemental PRL. At 6.5 dpc, 5 out of 9 *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ uteri displayed no implantation sites (Table 2-7). The other 4 females

showed small uterine swellings, but dye staining revealed abnormal vasculature (Fig. 2-5D1), indicative of decreased vascular permeability (Fig. 2-5C1). Examination of histological sections confirmed that PRL supplementation increased uterine cell density, but decidual tissue (Fig. 2-5D2) was not as fully developed as wild type or progesterone-rescued implantation sites (Fig. 2-5C2). Zero of four *Bmal1*$^{fx/fx}$*Cre*^{sf1} females supplemented with PRL until 10.5 dpc displayed uterine swelling (Table 2-8), but no developing embryos were observed (Fig. 2-5H). In summary, unlike progesterone, PRL could not completely rescue implantation failure in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females.

DISCUSSION

Circadian rhythms play a major role in mammalian reproduction. Female mice harboring a null allele at the *Bmal1* locus (i.e., *Bmal1*$^{-/-}$) display complete infertility. We chose to distinguish between two simplified models of how clocks might regulate reproduction in female mice. In one model, the master clock plays a primary role in reproduction through direct control of diurnal hormone secretion. Evidence for this model includes the observation that the master clock regulates the secretion of regulatory molecules such as vasopression (AVP), GnRH and kisspeptin (37). In turn, these molecules regulate LH and FSH and thus induce follicle development and ovulation. In an alternative model, the peripheral clocks of the reproductive axis play the primary role in female fertility. Evidence for this model includes CLOCK:BMAL1 enhancer elements in the promoter sequence of the *StAR* locus, which is consistent with the idea that the autonomous clocks within the gonads provide circadian control over steroidogenesis (2, 38, 39). In the present study, we employed a conditional knockout mouse model of *Bmal1*, coupled with ovary transplantation, to study the role of peripheral clocks in female fertility.

To delete *Bmal1* in steroidogenic tissues of the reproductive axis, we used the *Cre* transgene under the control of the *sf-1* promoter. The *Cre*$^{sf-1}$ transgene is well characterized, driving recombination within the anterior pituitary, adrenals, spleen and ovaries. While the activity of the *Cre*$^{sf-1}$ transgene has been seen in the ventromedial hypothalamic nucleus (VMH), it does not have activity in the SCN, the home of the master circadian clock (26, 40) (personal communication with Dr. Keith Parker). Our observation of complete infertility in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice provides eidence that a peripheral clock, likely in the steroidogenic cells within the

reproductive axis, is required for female fertility (Table 2-2). The *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females display implantation failure (Table 2-3, Fig. 2-5), recapitulating the phenotype of global *Bmal1*$^{-/-}$ females.

Our data are consistent with the idea that the luteal molecular clock is pivotal for implantation in female mice. Two lines of evidence support this conclusion. First, transplantation of wild type ovaries into *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females completely rescues birth rates (Table 2-6). Second, the observations that *Bmal1*$^{-/-}$ oocytes are fertilized and two-cell stage *Bmal1*$^{-/-}$ embryos transferred to wild type pseudopregnant females survive to weaning indicate that implantation failure in *Bmal1*$^{-/-}$ females is not significantly impacted by *Bmal1*$^{-/-}$ embryonic defects (Table 2-4). Our data also suggest that *Bmal1* regulates implantation through the control of progesterone levels. In support of this, *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females display significantly reduced progesterone levels, and administration of progesterone rescues implantation in *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females.

Indirect evidence also supports the importance of the ovarian clock for the implantation phenotype. Most importantly, we observed that implantation is not rescued by pituitary gonadotropins (FSH, LH) or PRL. PRL, a hormone secreted by the pituitary lactotrophs which supports luteinization in the rodent ovary, has been shown to be under transcriptional control by a molecular clock (41). However, the administration of PRL did not completely rescue implantation failure. Similarly, PMSG/HCG (which function as FSH and LH, respectively) could not reinstitute implantation sites in *Bmal1*$^{-/-}$ females (our unpublished data and (12)).

In an attempt to unravel the mechanisms underlying the role of the ovarian clock in implantation, we used microarrays to search for genes that were deregulated in *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$

ovaries at the time that implantation is initiated. Because the deregulated gene set was quite large (1605 transcripts with p-value < 0.02), we focused on the most significant subset of 73 highly significant genes (limma test p = 0.001). Interestingly, a global analysis of circadian dependent transcription reveals that only 10% of our subset are known to oscillate in SCN, pituitary, muscle, liver or cell culture in according to Circa, the circadian database http://bioinf.itmat.upenn.edu/circa/) (cosopt test pMMC-beta < 0.05, (42)). We interpret this as an indication that while *Bmal1* is primarily known for its role in molecular clock driven gene expression (Table 2-5B), its direct or indirect transcriptional targets are prevalent and also includes genes that do not display circadian rhythmicity. Pathway analysis of the 73 most significantly changed transcripts reveals that a disproportionate number of genes involved in the steroid biosynthetic pathway are dysregulated in *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ ovaries.

One of the most significantly downregulated genes arising in this analysis is *StAR*. The product of this locus is a mitochondrial cholesterol transporter that represents a rate-limiting step in progesterone biosynthesis (43). Attenuated *StAR* gene expression is also seen in the ovaries of *Bmal1*$^{-/-}$ female mice (13), and is consistent with the low serum progesterone levels observed in pregnant *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females (Fig. 2-4). These pieces of evidence suggest that the deletion of ovarian *Bmal1* results in lower progesterone secretion and thereby leads to the implantation failure in *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females. Several studies indicates that the expression of *StAR* is under regulation of BMAL1:CLOCK. First, the expression of *StAR* in F1 follicles of the chicken ovary circadian rhythm (38). Second, knock-down of *Bmal1* in the adrenal gland disrupts *StAR* expression and thus the rhythm of cortisol, a product of the steroid synthesis pathway in this tissue (2). Finally, chromatin immunoprecipitation assays and promoter analysis indicate that

Bmal1 binds to the distal E-boxes of the *StAR* promoter, and enhances transcription, suggesting direct regulation by the molecular clock (2, 39).

Another pathway revealed by our analysis is through G-protein coupled receptor protein signaling. Luteinizing hormone/choriogonadotropin receptor (*Lhcgr*) plays important roles in the formation and function of the corpus luteum. *Lhcgr* mRNA is down regulated in ovaries of $Bmal1^{fx/fx}Cre^{Sf\text{-}1}$ females, suggesting defects in luteinization and maintainance of luteal activity.

We suspect that the ability of 4 out of 11 $Bmal1^{fx/fx}$ females with $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ ovaries to produce litters is the result of progesterone production by residual wild type ovarian tissue to support implantation. In our experience, we found that the primary limitation of ovary transplantation is that it is difficult to remove all the recipient's ovaries, while maintaining sufficient integrity of the ovarian bursa to contain the donor ovaries. In support of this hypothesis, we found an intact follicle in the residual tissues of an intended recipient although no donor ovary had been transplanted.

To our knowledge, the $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ female is the only mouse model in which conditional ablation of a single clock gene results in complete infertility. Importantly, $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females are viable and exhibite none of the gross phenotypic abnormalities seen in $Bmal1^{-/-}$ females. They have no arthropathy (Fig. 2-2), signs of early aging, or body weight reduction. Moreover, they display normal body shape and tail length. While $Bmal1^{-/-}$ females displayed a trend toward lower rates of mating, $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females mate normally (Table 2-3). The absence of the many deficiencies displayed by global $Bmal1^{-/-}$ females helps us rule out potential unintended genetic deletions in off-target cell types. The complete infertility and implantation failure of $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ females, coupled with the rescue by ovary

transplantation, demonstrates that ovarian *Bmal1* is the primary core clock component controlling implantation in females. Furthermore, the mechanism by which this occurs is through regulation of luteal steroidogenesis in the ovary. $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ mice are a novel genetic model of reproduction that establishes essential roles of *Bmal1* in steroidogenesis and fertility.

REFERENCE

1. Boden MJ & Kennaway DJ (2006) Circadian rhythms and reproduction. *Reproduction* 132(3):379-392.
2. Son GH*, et al.* (2008) Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci U S A* 105(52):20970-20975.
3. Miller BH*, et al.* (2006) Vasopressin regulation of the proestrous luteinizing hormone surge in wild-type and Clock mutant mice. *Biol Reprod* 75(5):778-784.
4. Lowrey PL & Takahashi JS (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet* 5:407-441.
5. Labyak S, Lava S, Turek F, & Zee P (2002) Effects of shiftwork on sleep and menstrual function in nurses. *Health Care Women Int* 23(6-7):703-714.
6. Miller BH*, et al.* (2004) Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. *Curr Biol* 14(15):1367-1373.
7. Pilorz V & Steinlechner S (2008) Low reproductive success in Per1 and Per2 mutant mouse females due to accelerated ageing? *Reproduction* 135(4):559-568.
8. Bonnefont X (2010) Circadian timekeeping and multiple timescale neuroendocrine rhythms. *J Neuroendocrinol* 22(3):209-216.
9. Williams WP, 3rd, Jarjisian SG, Mikkelsen JD, & Kriegsfeld LJ (2011) Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology* 152(2):595-606.
10. Sellix MT & Menaker M (2011) Circadian clocks in mammalian reproductive physiology: effects of the "other" biological clock on fertility. *Discov Med* 11(59):273-281.
11. Yu EA & Weaver DR (2011) Disrupting the circadian clock: gene-specific effects on aging, cancer, and other phenotypes. *Aging (Albany NY)* 3(5):479-493.
12. Boden MJ, Varcoe TJ, Voultsios A, & Kennaway DJ (2010) Reproductive biology of female Bmal1 null mice. *Reproduction* 139(6):1077-1090.
13. Ratajczak CK, Boehle KL, & Muglia LJ (2009) Impaired steroidogenesis and implantation failure in Bmal1-/- mice. *Endocrinology* 150(4):1879-1885.
14. Williams DL & Schwartz MW (2005) Out of synch: Clock mutation causes obesity in mice. *Cell Metab* 1(6):355-356.
15. Fu L, Patel MS, Bradley A, Wagner EF, & Karsenty G (2005) The molecular clock mediates leptin-regulated bone formation. *Cell* 122(5):803-815.
16. Ando H*, et al.* (2011) Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice. *Endocrinology* 152(4):1347-1354.
17. Sadacca LA, Lamia KA, deLemos AS, Blum B, & Weitz CJ (2011) An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice. *Diabetologia* 54(1):120-124.
18. Bunger MK*, et al.* (2005) Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis* 41(3):122-132.

19. Andrews JL*, et al.* (2010) CLOCK and BMAL1 regulate MyoD and are necessary for maintenance of skeletal muscle phenotype and function. *Proc Natl Acad Sci U S A* 107(44):19090-19095.
20. Rudic RD*, et al.* (2004) BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* 2(11):e377.
21. Hemmeryckx B, Himmelreich U, Hoylaerts MF, & Lijnen HR (2011) Impact of clock gene Bmal1 deficiency on nutritionally induced obesity in mice. *Obesity (Silver Spring)* 19(3):659-661.
22. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, & Antoch MP (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* 20(14):1868-1873.
23. Marcheva B*, et al.* (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature* 466(7306):627-631.
24. Lee J*, et al.* (2011) Loss of Bmal1 leads to uncoupling and impaired glucose-stimulated insulin secretion in beta-cells. *Islets* 3(6).
25. Sun Y*, et al.* (2006) The mortality of MOP3 deficient mice with a systemic functional failure. *J Biomed Sci* 13(6):845-851.
26. Bingham NC, Verma-Kurvari S, Parada LF, & Parker KL (2006) Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44(9):419-424.
27. Allen E (1922) The estrous cycle in the mouse. *American journal of anatomy* 30(3):297-371.
28. Robb L*, et al.* (1998) Infertility in female mice lacking the receptor for interleukin 11 is due to a defective uterine response to implantation. *Nat Med* 4(3):303-308.
29. Brigid hogen RB, Frank Costantini, Elizabeth lacy (1994) Manipulating the Mouse Embryo: A Laboratory Manual (second edition).
30. Moons DS*, et al.* (2002) Pituitary hypoplasia and lactotroph dysfunction in mice deficient for cyclin-dependent kinase-4. *Endocrinology* 143(8):3001-3008.
31. Bachvarov D*, et al.* (2006) Renal gene expression profiling using kinin B1 and B2 receptor knockout mice reveals comparable modulation of functionally related genes. *Biol Chem* 387(1):15-22.
32. Sheward WJ*, et al.* (2007) Entrainment to feeding but not to light: circadian phenotype of VPAC2 receptor-null mice. *J Neurosci* 27(16):4351-4358.
33. Anonymous (!!! INVALID CITATION !!!).
34. Wang H & Dey SK (2006) Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* 7(3):185-199.
35. Daya S (2009) Luteal support: progestogens for pregnancy protection. *Maturitas* 65 Suppl 1:S29-34.
36. Huang da W, Sherman BT, & Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44-57.
37. Karatsoreos IN & Silver R (2007) Minireview: The neuroendocrinology of the suprachiasmatic nucleus as a conductor of body time in mammals. *Endocrinology* 148(12):5640-5647.
38. Nakao N*, et al.* (2007) Circadian clock gene regulation of steroidogenic acute regulatory protein gene expression in preovulatory ovarian follicles. *Endocrinology* 148(7):3031-3038.
39. Alvarez JD*, et al.* (2008) The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J Biol Rhythms* 23(1):26-36.
40. Caron KM, Clark BJ, Ikeda Y, & Parker KL (1997) Steroidogenic factor 1 acts at all levels of the reproductive axis. *Steroids* 62(1):53-56.
41. Leclerc GM & Boockfor FR (2005) Pulses of prolactin promoter activity depend on a noncanonical E-box that can bind the circadian proteins CLOCK and BMAL1. *Endocrinology* 146(6):2782-2790.

42. Hughes ME, Hogenesch JB, & Kornacker K (2010) JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. *J Biol Rhythms* 25(5):372-380.
43. Manna PR, Dyson MT, & Stocco DM (2009) Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. *Mol Hum Reprod* 15(6):321-333.

FIGURE LEGENDS

Figure 2-1. Generation and tissue-specific excision of the conditional *Bmal1*^{fx} allele. (*A*) Schematic diagram illustrating the region surrounding the basic-helix-loop-helix (bHLH) domain of the murine *Bmal1* locus. The targeted *Bmal1*^{fxneo} allele, the conditional *Bmal1*^{fx} allele, and the *Bmal1*^{fx}-excised allele are shown below. Exon numbers reflect known coding exons. The position of the inserted neomycin gene, flanked with *Frt* sites, and *loxP* sites flanking exon 4 are shown. Dotted lines indicate fragment sizes detected by the probe after digestion of genomic DNA with *Eco*RI. Oligonucleotides (OL) used for genotyping are indicated. (B) Southern blot of mouse tail biopsies showing bands of 5.1 and 6.9 Kbp, indicating the presence of the wild-type and *Bmal1*^{fxneo} allele, respectively. (C) To generate the conditional *Bmal1*^{fx} allele, the *Flp*^{Rosa} mouse was crossed to the *Bmal1*^{fxneo} mouse. Selective excision of the neomycin gene was detected through PCR genotyping of the region upstream of exon 4 that contained a *lox P* site and a *Frt* site using OL5439 and OL5440 as the forward and reverse primers, respectively. (D) PCR genotyping of the conditional *Bmal1*^{fx} allele. The primer pair OL6013/5436 detects the presence of the intact *lox P* site immediately downstream of exon 4 yielding a 350 bp product if present and a 300 bp product if absent. (E) Northern blot analysis of polyA RNA isolated from whole brain. Expression from the *Bmal1*$^{fx/fx}$ allele was similar to that of the wild-type allele. (F) Multiplex PCR analysis of *Bmal1*$^{fx/fx}$ excision in selective tissues from *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ mice. The primer pair OL6013/5436 amplifies the intact conditional alleles (~360 bp). The *Bmal1*^{fx}-excised allele is amplified with the OL6013/6014 primer pair and generates a ~450 bp band due to the loss of the floxed region and residual *lox P* and *Frt* sites remaining after excision. The

Bmal1^{fx}-excised allele is only detected in the brain, pituitary, adrenal gland, and ovaries and is absent in the liver, muscle, uterus and oviduct in *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females.

Figure 2-2. *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ mice exhibit normal circadian feeding rhythms with no arthropathy or reduction of body weight. (A) Food intake during the day (gray) and night (black) was measured in *Bmal1*$^{-/-}$ (n=2), wild type control(n=3, 1 *Bmal1*$^{fx/fx}$ and 2 *Bmal1*$^{+/+}$), and *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$(n=3) females. Values are shown as mean ±SEM over a period of 10 days. * P< 0.05 Wilcoxon rank sum test. (B) Hind limb tendons of 180 day old females stained with Alizarin Red show no evidence of the tendon calcification in *Bmal1*$^{+/+}$, *Bmal1*$^{fx/+}$*Cre*$^{sf\text{-}1}$ and *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$mice (arrows) that is present in *Bmal1*$^{-/-}$ mice. C. Body weights of wild type and *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females at 90 days and 210 days of age.

Figure 2-3. Effect of *Bmal1* deletion on the estrous cycle and ovaries. A. Representative patterns of estrous cycles from virgin *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ (n=6) and S-controls (*Bmal1*$^{fx/fx}$, n=4, *Bmal1*$^{fx/+}$cre$^{sf\text{-}1}$, n=1) females determined by vaginal cytology. Each day is represented by a diamond. C=cornified, N= nucleated, L= leukocytic. B. Representative pictures of ovaries from pregnant *Bmal1*$^{fx/+}$*Cre*$^{Sf\text{-}1}$ and *Bmal1*$^{fx/fx}$*Cre*$^{Sf\text{-}1}$ females displayed corpora lutea on 3.5 dpc. Black bars, 0.5mm.

Figure 2-4. Effect of *Bmal1* deletion on serum progesterone and PRL levels. Each point represents a single animal. A. progesterone levels at 3.5 dpc in *Bmal1*$^{+/+}$ (n=6), *Bmal1*$^{-/-}$(n=7), S-controls (*Bmal1*$^{fx/+}$*Cre*$^{sf\text{-}1}$, n=3, *Bmal1*$^{fx/fx}$, n=9), *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females (n=8). B. progesterone levels at 6.5 dpc from *Bmal1*$^{+/+}$ (n=5), *Bmal1*$^{-/-}$(n=6), S-controls (*Bmal1*$^{fx/+}$*Cre*$^{sf\text{-}1}$, n=2, *Bmal1*$^{fx/fx}$, n=7), *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females (n=14). C, PRL levels at ZT12 on 3.5 dpc listed in S-

control and *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females (n=8) at 3.5 dpc. D, PRL levels at ZT12 at 6.5 dpc from S-controls (n=9), *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females (n=14). P-value by wilcoxon rank sum test.

Figure 2-5. Effects of progesterone or PRL treatment of *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ and littermate control females on implantation at 6.5 dpc or 10.5 dpc. *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females (b,c,d,f,g,h) and wild type controls (a,e) received corn oil (a,b,e,f), progesterone (c,g),or prolactin (d,h) until 6.5 dpc (a,b,c,d) or 10.5 dpc (e,f,g,h). Pictures of representative whole uteri and H&E stained sections are shown. Saline, progesterone or PRL treated wild type controls are similar to corn oil treated wild type controls (pictures not shown). Scale bars, 0.5 mm.

TABLE LEGENDS

Table 2-1 oligo sequence

Table 2-2 *Bmal1*$^{-/-}$ and *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females are infertile. Fertility was scored as ability of a female to produce a litter within three months. Numbers in parentheses = number of mice tested. [a] Significantly different between mutant and controls ($p < 0.001$, Chi-square test) .

Table 2-3 Effect of *Bmal1* deletion on early pregnancy parameters.Values are given as proportion (n).

[a] Significantly different between mutant and controls (P<0.001, chi-square test).

[b] The mating rate is calculated as the number of females with plugs in the vagina/the total number of females mated (n).

[c] The ovulation rate was determined at 3.5 dpc and calculated as the number of females ovulated at 3.5 dpc/ the number of plugged females killed on 3.5dpc (n).

[d] The pregnancy rate was calculated as the number of females with detectable decidual swellings /the total number of 6.5dpc to 10.5 dpc females (n).

Table 2-4 *Bmal1*$^{+/+}$ and *Bmal1*$^{-/-}$ embryos were transferred into ICR pseudo pregnant dams. The survival rate is not significantly different between the two groups (Wilcoxon rank sum test). Experiment 6 came from cumulus data collected from our embryo transfer program in transgenic animal core facility.

Table 2-5 Microarray analysis revealed differently expressed genes in ovaries from *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females at 3.5 dpc.

Table 2-6 Rescue of *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ infertility by ovary transplantation

[a] Significantly different between mutants and their controls ($p<0.001$, chi square test)

Table 2-7 progesterone but not PRL rescued full implantation sites in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females at 6.5 dpc

[a] the number of implantation sites in mice with successful implantation. Values are Mean ± SEM.

b. $p<0.005$ vs. wild type (*Bmal1*$^{fx/wt}$*Cre*$^{sf-1}$or *Bmal1*$^{fx/fx}$) mice treated with corn oil (chi-square test).

[c] $p<0.005$ vs. *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice treated with corn oil (chi-square test).

[d] $p<0.001$ vs. wild type(*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$or *Bmal1*$^{fx/fx}$) mice treated with saline (chi-square test).

[e] $p=0.064$ vs. *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ mice treated with saline (chi-square test).

[f] Chicago sky blue staining incomplete/not permeated.

Table 2-8 progesterone but not PRL rescued implantation sites in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females till 10.5 dpc

[a] $p<0.05$ vs wild type (*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$or *Bmal1*$^{fx/fx}$) mice treated with corn oil (chi-square test).

[b] $p<0.005$ vs wild type (*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$or *Bmal1*$^{fx/fx}$) mice treated with saline (chi-square test).

[c] $P<0.05$ vs. wild type (*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$or *Bmal1*$^{fx/fx}$) mice treated with prolactin (chi-square test).

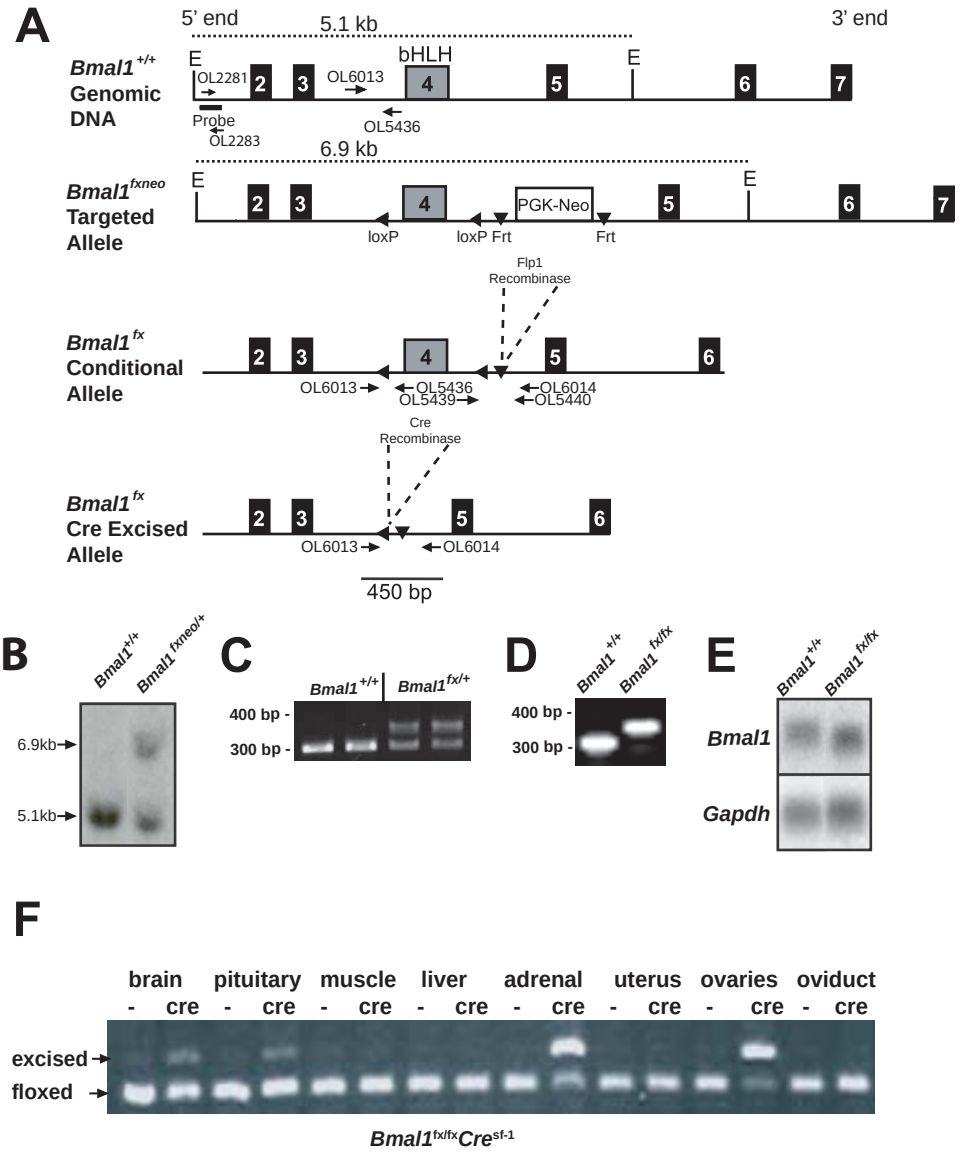


Figure 2-1

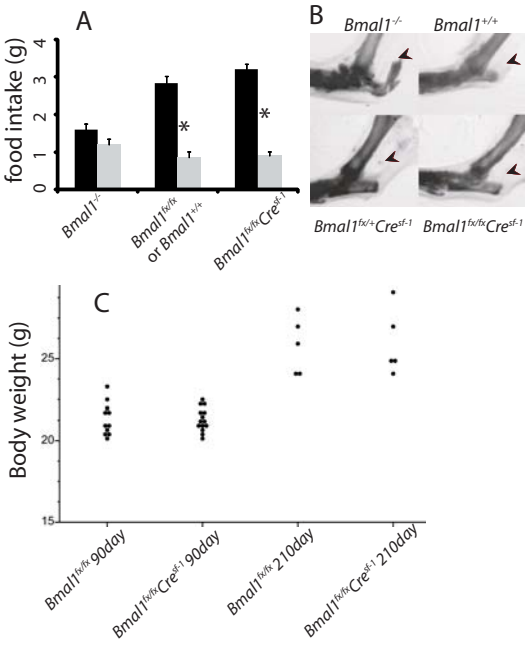


Fig. 2-2

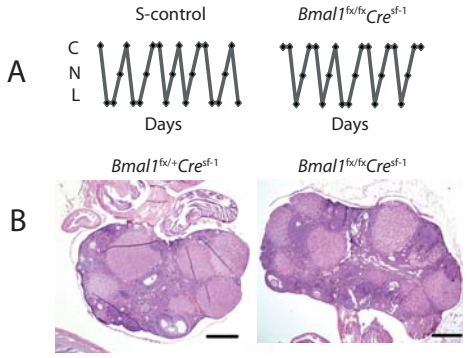


Fig. 2-3

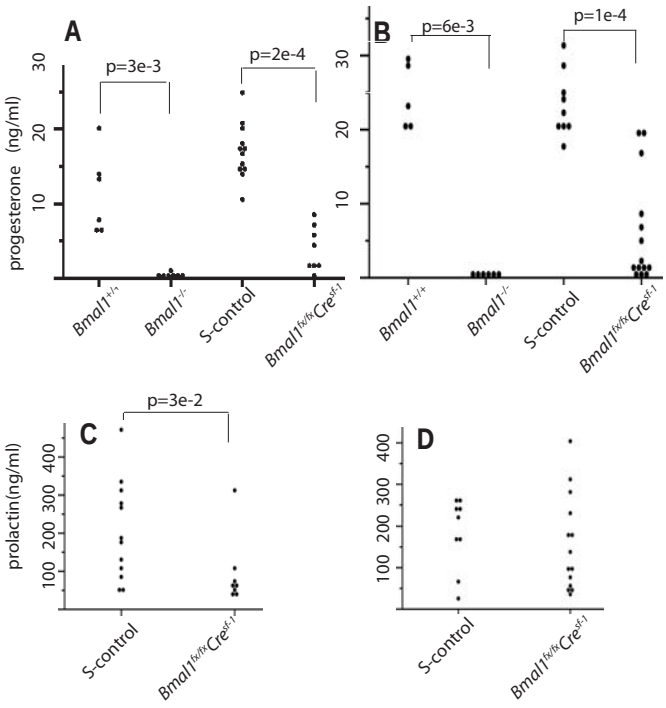


Figure 2-4

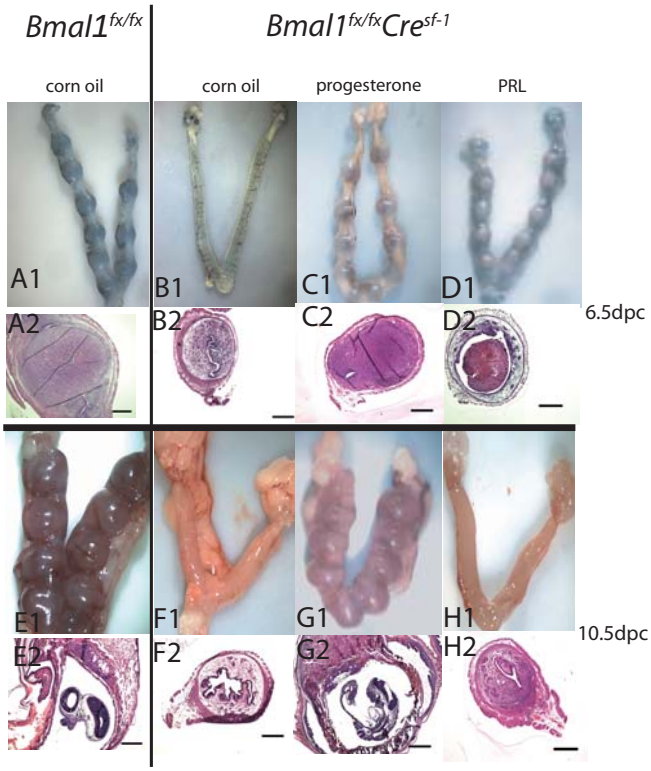


Fig 2-5

Table 2-1 oligo sequence

OL2281	5’-gct gtt ctg gat aaa tga tag
Ol2283	5’-ggc aaa acc ttc gag atg c
OL5439	5’-ctc atg tcc tct tgc ctg tg
OL5440	5’-ctt cca gtt tcc tgc ctc tg
OL5436	5’-ccc tga aca tgg gaa aga ga
OL6013	5’-att cac ctt ttg ggg agg ac
OL6014	5’-tca tca gag gaa cca ggg taa
OL2642	5’-tgc ctg cat tac cgg tcg atg c
OL2643	5’-cca tga gtg aac gaa cct ggt cg

Table 2-2 *Bmal1*$^{-/-}$ and *Bmal1*$^{fx/fx}$*Cre*$^{Sf-1}$ females are infertile

	Bmal1$^{+/+}$ (n=22)	*Bmal1*$^{-/-}$ (n=13)	*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ (n=23)	*Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ (n=12)
% of pregnancies reaching full term (proportion fertile/total)	95 (21/22)	0 [a]	96 (22/23)	0 [a]

Table 2-3 Effect of *Bmal1* deletion on early pregnancy parameters

	Bmal1$^{+/+}$	*Bmal1*$^{-/-}$	*Bmal1*$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$	*Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$
mating rate[b] in 14 days of breeding	0.88 (49)	0.81 (53)	0.9 (71)	0.93 (69)
Ovulation rate[c]	0.8 (15)	0.67 (27)	0.83 (41)	0.83 (42)
Pregnancy rate[d]	0.86 (28)	0 (16)[a]	0.83 (23)	0.048 (21)[a]

Table 2-4. *Bmal1*$^{-/-}$ or *Bmal1*$^{+/+}$ embryos transfer reveals similar survival rates					
	No. of embryos transferred		No. of pups born		Embryo survival rate
Experiment1	14	*Bmal1*$^{-/-}$	11	*Bmal1*$^{-/-}$	0.79
Experiment2	12	*Bmal1*$^{-/-}$	3	*Bmal1*$^{-/-}$	0.25
Experiment3	12	*Bmal1*$^{-/-}$	9	*Bmal1*$^{-/-}$	0.75
Experiment4	14	*Bmal1*$^{+/+}$	4	*Bmal1*$^{+/+}$	0.29
Experiment5	14	*Bmal1*$^{+/+}$	7	*Bmal1*$^{+/+}$	0.5
Experiment6	319	*Bmal1*$^{+/+}$	165	*Bmal1*$^{+/+}$	0.52

Table 2-5A. Differentially expressed genes in 3.5dpc *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ ovaries revealed by microarray analysis

gene name	Fold change	p.Value	description
Star	-17.70	4E-5	Steroidogenic acute regulatory protein(Star), mRNA [NM_011485]
Cldn18	-15.23	4E-5	claudin 18 (Cldn18), mRNA [NM_019815]
Vnn3	-7.47	1E-4	vanin 3 (Vnn3), mRNA [NM_011979]
Clic3	-5.12	3E-4	chloride intracellular channel 3 (Clic3), mRNA [NM_027085]
Cela1	-4.80	1E-3	10 day old male pancreas cDNA, RIKEN full-length enriched library, clone:1810062B19 product:elastase 1,
5330417C22Rik	-4.52	4E-4	RIKEN cDNA 5330417C22 gene, mRNA (cDNA clone IMAGE:4502533), partial cds [BC051424]
CJ042244	-4.24	2E-5	CJ042244 RIKEN full-length enriched mouse cDNA library, C57BL [CJ042244]
Bpil3	-3.86	8E-4	bactericidal/permeability-increasing protein-like 3 (Bpil3), mRNA [NM_199303]
Tm7sf2	-3.71	5E-4	transmembrane 7 superfamily member 2 (Tm7sf2), mRNA [NM_028454]
D630042P16Rik	-3.23	7E-4	2 days pregnant adult female ovary cDNA, RIKEN full-length enriched library, clone:E330001L02 product:s
Tcap	-3.21	7E-4	titin-cap (Tcap), mRNA [NM_011540]
Sez6l2	-2.95	4E-4	seizure related 6 homolog like 2 (Sez6l2), mRNA [NM_144926]
Dbp	-2.87	4E-5	D site albumin promoter binding protein (Dbp), mRNA [NM_016974]
Pex1	-2.67	6E-4	peroxisome biogenesis factor 1 (Pex1), mRNA [NM_027777]
Oxtr	-2.64	9E-4	Mus sp. mRNA for oxytocin receptor, complete cds. [D86599]
Fam195a	-2.62	6E-4	RIKEN cDNA 9530058B02 gene (9530058B02Rik), mRNA [NM_026633]
Dhcr7	-2.58	6E-4	7-dehydrocholesterol reductase (Dhcr7), mRNA [NM_007856]
Lhcgr	-2.40	8E-5	luteinizing hormone/choriogonadotropin receptor (Lhcgr), mRNA [NM_013582]
Pmvk	-2.18	5E-4	phosphomevalonate kinase (Pmvk), mRNA [NM_026784]
Tbxa2r	-2.16	8E-4	thromboxane A2 receptor (Tbxa2r), mRNA [NM_009325]
Hp	-2.10	8E-4	haptoglobin (Hp), mRNA [NM_017370]
AK040678	-2.10	5E-4	adult male aorta and vein cDNA, RIKEN full-length enriched library, clone:A530014A01 product:natriuretic
Bace2	-2.09	4E-4	beta-site APP-cleaving enzyme 2 (Bace2), mRNA [NM_019517]
Acat3	-1.97	3E-4	acetyl-Coenzyme A acetyltransferase 3 (Acat3), mRNA [NM_153151]
Alox12	-1.93	9E-4	arachidonate 12-lipoxygenase (Alox12), mRNA [NM_007440]
Usp39	-1.85	7E-4	ubiquitin specific peptidase 39 (Usp39), mRNA [NM_138592]
Tmem97	-1.84	3E-4	transmembrane protein 97 (Tmem97), mRNA [NM_133706]
AK013921	-1.84	4E-4	13 days embryo head cDNA, RIKEN full-length enriched library, clone:3100002L24 product:similar to KRU
Tlcd1	-1.82	2E-4	TLC domain containing 1 (Tlcd1), mRNA [NM_026708]
Tbl2	-1.75	1E-3	transducin (beta)-like 2 (Tbl2), mRNA [NM_013763]
Pnmt	-1.72	1E-3	phenylethanolamine-N-methyltransferase (Pnmt), mRNA [NM_008890]
Slc35e3	-1.71	7E-4	solute carrier family 35, member E3 (Slc35e3), mRNA [NM_029875]
Med13	-1.69	1E-4	PREDICTED: Mus musculus thyroid hormone receptor associated protein 1, transcript variant 1 (Thrap1), m
2010001M09Rik	-1.65	3E-4	RIKEN cDNA 2010001M09 gene (2010001M09Rik), mRNA [NM_027222]
Ly6c1	-1.57	2E-4	lymphocyte antigen 6 complex, locus C (Ly6c), mRNA [NM_010741]
ENSMUST00000045536	-1.55	2E-4	10 day old male pancreas cDNA, RIKEN full-length enriched library, clone:1810047P18 product:hypothetica
NAP001160-001	-1.55	3E-4	Unknown
Gm166	-1.49	7E-4	gene model 166, (NCBI) (Gm166), mRNA [NM_001033040]
Amica1	-1.48	9E-4	adhesion molecule, interacts with CXADR antigen 1 (Amica1), mRNA [NM_001005421]
Cox8b	1.59	6E-4	cytochrome c oxidase, subunit VIIIb (Cox8b), mRNA [NM_007751]
Dpy19l3	1.59	9E-4	dpy-19-like 3 (C. elegans) (Dpy19l3), mRNA [NM_178704]
Tlr2	1.63	1E-3	toll-like receptor 2 (Tlr2), mRNA [NM_011905]
St3gal1	1.64	2E-4	ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (St3gal1), mRNA [NM_009177]
TC1502037	1.67	3E-4	Q8JZS5 (Q8JZS5) Filamin-interacting protein S-FILIP, partial (27%) [TC1502037]
Lca5l	1.67	1E-4	RIKEN cDNA 4921526F01 gene (4921526F01Rik), mRNA [NM_001001492]
Gab3	1.67	4E-4	growth factor receptor bound protein 2-associated protein 3 (Gab3), mRNA [NM_181584]
Gas2l1	1.71	3E-4	M.musculus mRNA polyA site sequence. [M89786]
Gpr171	1.71	2E-4	G protein-coupled receptor 171 (Gpr171), mRNA [NM_173398]
BC030336	1.75	5E-4	ENSMUST00000098077 [BC030336]
Srxn1	1.91	8E-4	sulfiredoxin 1 homolog (S. cerevisiae) (Srxn1), mRNA [NM_029688]
Spag4	2.01	2E-4	sperm associated antigen 4 (Spag4), mRNA [NM_139151]
Saa2	2.01	1E-3	serum amyloid A 2 (Saa2), mRNA [NM_011314]

Cd83	2.17	5E-4	CD83 antigen (Cd83), mRNA [NM_009856]
Nuak1	2.18	5E-5	adult male corpora quadrigemina cDNA, RIKEN full-length enriched library, clone:B230104P22 product:unc
Gng2	2.35	9E-4	guanine nucleotide binding protein (G protein), gamma 2 subunit (Gng2), transcript variant 1, mRNA [NM_0
Procr	2.41	4E-4	protein C receptor, endothelial (Procr), mRNA [NM_011171]
Dmrta1	2.77	8E-4	doublesex and mab-3 related transcription factor like family A1 (Dmrta1), mRNA [NM_175647]
Ccrl2	2.78	9E-4	chemokine (C-C motif) receptor-like 2 (Ccrl2), mRNA [NM_017466]
Insrr	2.84	3E-4	insulin receptor-related receptor (Insrr), mRNA [NM_011832]
Tubb6	3.01	1E-3	tubulin, beta 6 (Tubb6), mRNA [NM_026473]
Lrp11	3.43	6E-4	low density lipoprotein receptor-related protein 11 (Lrp11), mRNA [NM_172784]
Adm	4.54	7E-5	adrenomedullin (Adm), mRNA [NM_009627]
D630023F18Rik	4.56	5E-4	RIKEN cDNA D630023F18 gene (D630023F18Rik), mRNA [NM_175293]
Fam84a	4.71	7E-4	expressed sequence AW125753 (AW125753), mRNA [NM_029007]
Egln3	5.32	1E-3	EGL nine homolog 3 (C. elegans) (Egln3), mRNA [NM_028133]
Adra2a	5.44	5E-5	adrenergic receptor, alpha 2a (Adra2a), mRNA [NM_007417]
Ermp1	5.73	1E-3	DNA segment, Chr 19, Wayne State University 12, expressed, mRNA (cDNA clone IMAGE:3987774), comp
Adra2c	6.06	3E-4	adrenergic receptor, alpha 2c (Adra2c), mRNA [NM_007418]
Kcnmb4	7.11	2E-4	potassium large conductance calcium-activated channel, subfamily M, beta member 4 (Kcnmb4), mRNA [NM
Tmem26	9.19	3E-5	transmembrane protein 26 (Tmem26), mRNA [NM_177794]
4933400F03Rik	10.84	8E-4	adult male testis cDNA, RIKEN full-length enriched library, clone:4933400F03 product:hypothetical Arginin
Corin	11.53	2E-4	corin (Corin), mRNA [NM_016869]
Galntl5	12.62	3E-4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-like 5 (Galntl5), mRN

Table 2-5B Deregulated core clock genes in 3.5dpc *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ovaries revealed by microarray analysis

	Fold change	P Value	description
Cry1	2.65	3E-2	Mus musculus cryptochrome 1 (photolyase-like) (Cry1), mRNA [NM_007
Dbp	-2.86	4E-5	D site albumin promoter binding protein (Dbp), mRNA [NM_016974]
Npas2	4.38	1E-2	Mus musculus neuronal PAS domain protein 2 (Npas2), mRNA [NM_008
Nr1d1	-3.45	1E-2	Mus musculus nuclear receptor subfamily 1, group D, member 1 (Nr1d1), [NM_145434]
Nr1d2	-4.50	3E-3	Mus musculus nuclear receptor subfamily 1, group D, member 2 (Nr1d2), [NM_011584]
Rora	2.29	7E-3	Mus musculus RAR-related orphan receptor alpha (Rora), mRNA [NM_01

Table 2-5C Pathways affected in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ ovaries revealed by the "DAVID Bioinformatics"

term	P value
GO:0007186~G-protein coupled receptor protein signaling pathway	4E-3
GO:0006694~steroid biosynthetic process	7E-3
GO:0006695~cholesterol biosynthetic process	1.3E-2
GO:0016126~sterol biosynthetic process	1.8E-2
GO:0008202~steroid metabolic process	3.4E-2

Table 2-6 Rescue of *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ infertility by ovary transplantation		
Genotype of donor ovary	*Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ (n=11)	*Bmal1*$^{fx/fx}$ (n=12)
Recipient genotype	*Bmal1*$^{fx/fx}$ (n=11)	*Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ (n=12)
Number of transplanted females producing live ltters	4	12
Proportion of fertility	0.36	1[a]

Table 2-7 P4 but not PRL rescues implantation in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females at 6.5 dpc			
Mice genotype	No. of mice tested	% of mice with implantation	No. of implantation sites/mouse[a]
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ + corn oil	11	91	9.4±0.4
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ + P4	12	75	8.9±0.4
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$ +corn oil	9	0[b]	0
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$ + P4	8	62.5[c]	7.2±1.2
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ + saline	11	91	8.8±0.33
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ + PRL	8	87.5	9.7±0.43
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$+saline	6	0[d]	0
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$ +PRL	9	44[e,f]	8±0.91

Table 2-8 P4 but not PRL rescues implantation in *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ females on 10.5dpc			
Mice	No. of mice tested	% of mice with implantation	No. of implantation sites
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ + corn oil	2	100	9,9
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or *Bmal1*$^{fx/fx}$ + P4	2	100	9,8
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$+cornoil	2	0[a]	0,0
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$ +P4	5	60	0,0,5,9,11
Bmal1$^{fx/+}$*Cre*$^{sf-1}$or*Bmal1*$^{fx/fx}$ + saline	4	100	6,8,9,10
Bmal1$^{fx/+}$*Cre*$^{sf-1}$ or*Bmal1*$^{fx/fx}$ + PRL	1	100	9
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$+saline	3	0[b]	0
Bmal1$^{fx/fx}$*Cre*$^{sf-1}$ +PRL	4	0[c]	0

CHAPTER III

Genetic dissection suggests that *Bmal1* in different cell types contribute to male and female fertility in mice

ABSTRACT

Circadian rhythms are reported to regulate reproductive physiology. Both *Bmal1*$^{-/-}$ male and female mice display a subfertility phenotype likely due to impaired steroidogenesis in the testis and ovaries in males and females respectively (1, 2). In our study we employed Cre-LoxP systems including Sf-1/cre and Nestin/Cre to target *Bmal1* deletions to steroidogenic cells of the reproductive axis or cells of the central nervous system, respectively. The central nervous system conditional knockout *Bmal1*$^{fx/fx}$*Cre*^{nestin} males have compromised fertility with smaller size of testis and seminal vesicles. No copulation plugs were found in the female partners in the breedings involving the infertile *Bmal1*$^{fx/fx}$*Cre*^{nestin} males. The peripheral reproductive axis conditional knockout *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ male mice are fertile without detectable reproductive defects. In contrast, the conditional deletions produce opposite phenotypes in females. *Bmal1*$^{fx/fx}$*Cre*^{nestin} females are fertile while *Bmal1*$^{fx/fx}$*Cre*$^{sf\text{-}1}$ females are infertile. We conclude that *Bmal1* in different cell types contributes to male and female fertility in mice. Disruption of *Bmal1*$^{-/-}$ in the central nervous system contributes to the compromised male fertility while the *Bmal1* disruption in the reproductive axis especially the ovaries results in the infertility in female mice.

INTRODUCTION

Infertility affects approximately every one out of six couples (3). We propose that the modern environment (such as light/dark patterns) and lifestyle factors may affect human health and reproductive outcome. Among those, chronic light exposure leading to disrupted circadian rhythms is known to have adverse association with reproductive fitness. Similarly, there is strong epidemiological evidence indicating that shift work is associated with menstrual irregularities and reproductive disturbances (4).

In mammals, circadian physiology is controlled by a molecular clock that is present in virtually every cell type. At the core of this molecular clock is the CLOCK: BMAL1 (also known as ARNTL and MOP3) dimer that transcriptionally activates feed-back components, such as those encoded by *Per*1-3, *Cry*1-2, *Rorα*, *Nr1d1 and Nr1d2* genes. Additional "output" genes important in regulating circadian physiology include *vasopressin (Avp), vasoactive intestinal peptide (Vip), neuropeptide Y (NPY), Leptin receptor (lepR), and steroidogenic acute regulatory protein* (*StAR*) (5-8).

The circadian system regulates daily temporal organization in behavior and neuroendocrine rhythms through both central clocks and cell-specific peripheral clocks. The cells in suprachiasmatic nuclei (SCN) act as a synchronizer of peripheral cellular clocks (9). Within the brain, SCN projections to the preoptic area (POA) provide control over the timing of the release of gonadotrophin-releasing hormone (GnRH) (10, 11). In addition to SCN, *Bmal1* is expressed in many extra-SCN sites in both males and females. BMAL1 and PER2 proteins cycle in the GnRH neurons in the POA, medial septum (MS), anterior hypothalamic area (AHA), and the lateral hypothalamus (LHA) (12, 13). *Bmal1* also expresses in the olfactory bulb, olfactory

cortex, retina, paraventricular nucleus (PVN), arcuate nucleus of the hypothalamus (ARC), and the dorsomedial hypothalamic nuclei (DMH), all of which could contribute to various aspects of fertility such as mating behavior, feeding behavior and neuroendocrinology (14, 15). For example, hypothalamic *NPY* and *lepR* exhibit circadian expression and are implicated to serve as a communication bridge to link reproduction, behavior, feeding, and energy homeostasis (16-21).

Peripheral endocrine tissues are also influenced by circadian rhythms of clocks. In lactotrophs and gonadotrophs of the pituitary, BMAL1: CLOCK dimer can drive the expression of *prolactin* (*PRL*) and *GnRH receptor* (*Gnrhr*) through binding at E-box elements of their promoters (22, 23). In the adrenal gland, *Bmal1*autonoumously confers the circadian expression of *StAR*, the rate-limiting steroidogenic factor, and thus drives the diurnal variations of serum cortisol (24). Similarly, pancreatic *Bmal1* is required for normal insulin release(25). Plasma leptin and leptin mRNA in adipose tissue display circadian rhythms (26). In testis although circadian rhythm of *Bmal1* mRNA expression has not been shown, daily variations in BMAL1 protein expression in leydig cells has been reported (1, 27). In ovaries, *Bmal1* is required for steroidogenesis (chapter 1)(2).

Mouse models carrying mutations in circadian genes reveals the relationship between circadian clocks and fertility. In this regard, global *Bmal1* knockout (*Bmal1*$^{-/-}$) mice display infertility in both males and females. Low testosterone and progesterone levels due to reduced expression of *StAR* in testis or ovaries were suggested to be a main cause for male and female infertility respectively (1) (2).

Although *Bmal1*$^{-/-}$ seems to be the most powerful circadian mutant model to study the relationship between reproductive biology and circadian biology, the global deletion of the locus

also leads to many non-reproductive phenotypes such as arthropathy, diabetes, and early aging. These extreme phenotypes can complicate the interpretation of the role of *Bmal1* in reproduction. In order to study the relative roles of different clocks in male and female fertility we have employed the Cre-loxP system to genetically delete *Bmal1* in a number of candidate cell types including the central nervous system, the steroidogenic cells, the pit-1 cell lineages (somatotroph, lactotroph and thyrotrophs), granulosa cells, and leydig cells (table 3-1) . We found that *Bmal1* in the reproductive axis (including hypothalamic-pituitary-gonad (HPG) axis) is necessary for full female fertility while *Bmal1* in the central nervous system is the more significant contributor to male subfertility.

MATERIALS AND METHODS

Gene targeting and animal husbandry.

A description of the generation of a *Bmal1* allele that is flanked by lox P sites (floxed) is shown in Chapter 2. To disrupt the circadian clock in specific cell types, mice harboring the $Bmal1^{fx/fx}$ allele were crossed to mice expressing a *Cre* transgene driven by various cell specific promoters (Table 3-1). All breedings were set up so that *Cre* transgenes exist either in sire or dam but never both, so that all offspring carry only one copy of *Cre*.

To disrupt the circadian clock in the reproductive axis, mice harboring the $Bmal1^{fx}$ allele were bred to mice expressing a *Cre* transgene driven by the S*f-1* (also as *Nr5a1*) promoter (28, 29). The Sf-1/cre mouse line (a generous gift from Keith L Parker, University of Texas Southwestern, hereafter referred to as $Cre^{sf\text{-}1}$) was bred to C57BL/6J background for more than 7 generations (N>7). These $Cre^{sf\text{-}1}$ mice are outwardly healthy and bred well. $Cre^{sf\text{-}1}$conveys recombination in the somatic cells of the gonads (Leydig cells and Sertoli cells in male mice, granulosa cells in female mice), the adrenal cortex, the anterior pituitary (gonadotrophs), the spleen, and the ventromedial hypothalamic nucleus as early as 10.5 dpc (29).

To disrupt the circadian clock in the central nervous system, mice harboring the $Bmal1^{fx}$ allele were bred to mice expressing a *Cre* transgene driven by the rat *Nestin* promoter (30). The B6.Cg-Tg(Nes-cre)1Kln/J mice (purchased from the Jackson Laboratory, Bar Harbor, Maine, USA) (here after Cre^{nestin}) were bred to C57BL/6J background for more than 10 generations (N>10). Cre^{nestin} is used to conditionally to knock out gene expression in the central nervous system in many studies (30) (31).

To disrupt the circadian clock in pit-1 cell lineages (including growth hormone producing somatotrophs, PRL producing lactotrophs, and TSH producing thyrotrophs) (32) mice harboring the *Bmal1*^{fx} allele were bred to mice expressing a *Cre* transgene driven by the rat growth hormone releasing hormone receptor promoter. The rGhrhr/cre (a generous gift from Dr. Kirschner LS, Ohio State University, hereafter referred to as *Cre*^{rGhrhr}) mice were bred to C57BL/6J background for 4 generations (N=4 on C57BL/6J background).

To disrupt the circadian clock in leydig cells (testosterone producing cells) mice harboring the *Bmal1*^{fx} allele were bred to mice expressing a *Cre* transgene driven by Amhr2 promoter. Amhr2/cre (hereafter referred to as *Cre*^{Amhr2}). *Cre*^{Amhr2} mice were purchased from Mutant Mouse Regional Resource Centers in NCRR-NIH, Chapel Hill, North Carolina). Mice were bred to C57BL/6J background for more than 6 generations (N>6 on C57BL/6J background). Cre^{Amhr2} mice have been used as leydig cell-specific conditional deleter in male mice (33) and granulosa cell-specific conditional delete in female mice (34).

In breedings involving Cre^{sf-1} or Cre^{rGhrhr}, we havn't detected germ line activation in Cre^{sf-1} or Cre^{rGhrhr} based on the fact that there were no excised alleles in mice without Cre transgene). Mice homozygous for the floxed allele and hemizygous for Cre were used for experiments. details, *Bmal1*$^{fx/fx}$Cre^{sf-1} (here after S-*Bmal*$^{-/-}$) and *Bmal1*$^{fx/fx}$*Cre*^{rGhrhr} (hereafter referred to as G-*Bmal1*$^{-/-}$) mice are used as reproductive axis and somatotroph/lactotroph conditional knockouts respectively. Mice that positive for the C*re* transgene while carrying a wild type allele *Bmal1*$^{fx/+}$Cre^{sf-1}(hereafter referred to as S-control), or *Bmal1*$^{fx/+}$Cre^{rGhrhr} (hereafter referred to as G-control) were used as wildtype controls. In breedings involving Cre^{nestin} and Cre^{Amhr2} we have detected germ line *Cre* activation based on the detection of excised allele without Cre. *Bmal1*$^{fx/fx}$*Cre*^{nestin} and *Bmal1*$^{fx/ex}$*Cre*^{nestin} (hereafter referred to as N-*Bmal1*$^{-/-}$) mice are used as

central nervous system conditional knockouts. Mice that were positive for the C*re* transgene while having a wild type allele (*Bmal1*$^{ex/+}$Cre^{nestin} or *Bmal1*$^{fx/+}$Cre^{nestin}) were used as normal controls (hereafter referred to as N-control). *Bmal1*$^{fx/fx}$*Cre*^{Amhr2} and *Bmal1*$^{fx/ex}$*Cre*^{Amhr2} (hereafter referred to as A-*Bmal1*$^{-/-}$) mice are used as leydig cell or granulose cell conditional knockouts. Mice that were positive for the C*re* transgene while carrying a wild type *Bmal1*^{fx} allele (*Bmal1*$^{ex/+}$Cre^{Amhr2} or *Bmal1*$^{fx/+}$*Cre*^{Amhr2}) were used as normal controls (hereafter referred to as A-control).

All procedures were approved by the Animal Care and Use Committee, University of Wisconsin. Mice were housed under normal 12hr: 12hr light: dark cycle with standard lab chow (Mouse diet 9F 5020, PMI Nutrition International, LLC, Brentwood, MO 63144) and water ad libitum.

Male fertility assessment.

Male fertility was tested by pairing a single 8 week old S-*Bmal*$^{-/-}$, G-*Bmal*$^{-/-}$, A-*Bmal*$^{-/-}$, or N-*Bmal*$^{-/-}$ male mouse with a wild type female mouse (aged between 9 and 16 weeks old) for at least 2 months. Cages were inspected daily for presence of distended abdomen or pups. Male fertility was defined as production of at least one litter in this period.

Female fertility assessment.

Eight week old S-*Bmal*$^{-/-}$, G-*Bmal*$^{-/-}$, A-*Bmal*$^{-/-}$, or N-*Bmal*$^{-/-}$ females were paired with wild type male of known fertility for at least 2 months. The S-*Bmal*$^{-/-}$ females were mated to wild type males of known fertility for 110 to 130 days. Female fertility was defined as production of at least one litter in this period.

Hormone measurement.

90-130 day old mice were euthanized with carbon dioxide gas. Blood was collected from the hearts and allowed to coagulate at room temperature for an hour before centrifugation. Serum was separated by centrifugation at 10000 min^{-1} for 10 minutes and stored at-30^{o}C until assayed. Testosterone levels were analyzed by Radioimmunoassay in the laboratory of National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, California).

Semen analysis.

Semen analysis was done by Dr. Brad Didion at Minitube of America (Mount Horeb, Wisconsin). Briefly 19-22 week old *Bmal1*$^{-/-}$ *or Bmal1*$^{+/+}$ males were euthanized by carbon dioxide. The epididymides were isolated and placed in a 200 µl droplet of KSOM + 30mg/ml BSA buffer at room temperature (35). Using fine forceps to mince the cauda epididymis and incubation at 37^{o}C for 20 minutes for dispersion. A computer assisted sperm analysis program (SpermVision, Minitube Inc.) was used to evaluate the motility (36).

Histological analysis.

For histological characterization, tissues were fixed in 10% v/v buffered formalin over night, dehydrated in graded ethanol and embedded in paraffin. Sections (7µm) were stained with hematoxylin and eosin (H&E) and analyzed under light microscopy as described (1).

Genotyping, assessment of estrous cycle, assessment of feeding behavior.

These methods are described in chapter two.

Statistical analyses.

The Wilcoxon rank sum test (two-sided unless indicated) was used in all hypotheses testing for difference in locations. The Chi-square test was consulted in all hypotheses testing for difference in proportion (Statistical problems in genetics and molecular biology, Norman Drinkwater and Carter Denniston).

RESULTS

Conditional *Bmal1*$^{-/-}$ knockout mice enable evaluation of peripheral clocks in fertility. In order to evaluate the importance of cell-specific circadian clocks in male and female fertility, we employed four *Cre* transgenic lines listed in Table 3-1. We characterized the tissue-specific deletion of *Bmal1* in S-*Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ mice using PCR amplification of genomic DNA from various tissues. As shown in Figure 3-1, in S-*Bmal1*$^{-/-}$ males *Bmal1* was partially excised in the brain, pituitary, adrenal gland, and testis but not in liver, muscle, and seminal vesicles. This pattern is consistent with the expression pattern of the *sf-1* gene (29). In contrast to S-*Bmal1*$^{-/-}$ males, *Bmal1* was almost completely deleted in the brain of N-*Bmal1*$^{-/-}$ mice. Partial excision was observed in pituitary, and muscle of both sexes, and in ovaries in females or testis in males. This pattern is consistent with previous reports of *Cre*^{nestin} mice (37-39).

The age associated arthropathy reported in global *Bmal1*$^{-/-}$ animals is not observed in S-*Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ mice. Unlike the *Bmal1*$^{-/-}$ mice, S-*Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ mice could survive normally for greater than 6 months (40). To further exclude the possibility that the arthropathy caused physical hindrance of copulatory activity that could contributes to infertility, hind limbs of 6 month old S-*Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ mice were stained with alizarin red (40) to detect calcification of ligaments and tendons. As shown in Figure 3-2, none of the S-*Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ mice displayed any abnormal visible calcification. Consistently, S-*Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ mice moved freely and flexibly without any stiffness.

***N-Bmal1*$^{-/-}$ males have compromised fertility while S-*Bmal*$^{-/-}$, A-*Bmal*$^{-/-}$ *and* G- *Bmal*$^{-/-}$ males display normal fertility.**

As shown in table 3-2, 19 of 20 *Bmal1*$^{-/-}$ males and 9 out of 17 N-*Bmal1*$^{-/-}$ males did not sire any litters in two months' breeding ($P<0.05$, chi-square test). In contrast, 17 out 17 S-

Bmal1$^{-/-}$ males, 6 out of 6 A-*Bmal1*$^{-/-}$ and 5 out of 5 G-*Bmal1*$^{-/-}$ sired litters. There were no significant differences in litter size between all fertile conditional knockout males and the control normal males.

In order to evaluate the mating behavior of *Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ males, we followed the female partners of 11 *Bmal1*$^{-/-}$ and 4 N-*Bmal1*$^{-/-}$ males using vaginal plug checking. Plugs were detected in female partners of 7 of 7 wild type males and 7 or 7 S-Bmal males within 6 days of mating. However, in 14 days, only 1 plug was found in the 11 matings involving the *Bmal1*$^{-/-}$ males. No plugs were found in the female partners of the other 10 *Bmal1*$^{-/-}$ males. Similarly no plugs were found in the female partners of the 4 infertile N-*Bmal1*$^{-/-}$ males. To rule out fertility problems with the wild type female partner, the 9 infertile N-*Bmal1*$^{-/-}$ males described above were mated with multiple wild type females for a period for at least 3 months. Except one infertile N-*Bmal1*$^{-/-}$ sired a litter three months later, the other 8 did not sire any litters.

S-*Bmal1*$^{-/-}$ females are infertile while N-*Bmal1*$^{-/-}$, A-*Bmal1*$^{-/-}$and G-*Bmal1*$^{-/-}$ females are fertile.

As shown in table 3-3, none of the 12 S-*Bmal1*$^{-/-}$ females mated with wildtype males gave birth to any litters in breeding although plugs were found every 6-8 days (the same 12 females in chapter 2). In contrast, N-*Bmal1*$^{-/-}$, A-*Bmal1*$^{-/-}$and G-*Bmal1*$^{-/-}$ females are fertile with normal gestation time (19.5-20.5days) and normal litter size (4-10 pups/litter).

N-*Bmal1*$^{-/-}$ females display defects in some aspects of reproduction.

Although *N-Bmal1*$^{-/-}$ females were able to carry pregnancies to full term, they did not bring litters to weaning stage as well as N-control females. In 55 closely observed litters (17 first litters, 18 second litters, 13 third litters,7 fourth litters) from *N-Bmal1*$^{-/-}$ females, only 12 litters (2

first litters, 4 second litters, 2 third litters, 4 fourth litters) survived to weaning (i.e., 21 days after birth). The remaining 43 litters died 1-2 days after birth. In those 12 litters scored as "survival to weaning", about half of pups died during the weaning process. In contrast, in 30 litters (10 first litters, 8 second litters, 6 third litters, 3 fourth litters, 2 fifth litters and 1 sixth litter) delivered by N-control females 18 litters (3 first litters, 5 second litters, 5 third litters, 2 fourth litters, 2 fifth litters and 1 sixth litter) survived to weaning. The rate of postnatal survival to weaning is statistically higher in N-control dams than N-*Bmal1*$^{-/-}$ dams ($p=0.0004$, chi-square test).

Virgin N-*Bmal1*$^{-/-}$ females also display a higher frequency of irregular estrous cycles ($P<0.05$, Wilcoxon rank sum test). As shown in Figure 3-3, wild type control mice have 1-2 days of diestrus followed by 1-2 days of proestrus and then 1-3 days of estrus. In contrast, among 12 virgin N-*Bmal1*$^{-/-}$ females, 2 displayed more than 8 days of continuous diestrus and 2 displayed more than 5 days of continuous estrus.

***Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ males have smaller size of testis and seminal vesicles.**

As shown in Table 3-4, Figures 3-4 and 3-5, *Bmal1*$^{-/-}$ males have smaller testes, and seminal vesicles than *Bmal1*$^{+/-}$ and *Bmal1*$^{+/+}$ males. Similarly N-*Bmal1*$^{-/-}$ males have smaller testes and seminal vesicles than N-controls. Although N-control males have smaller seminal vesicles and testis than *Bmal1*$^{fx/fx}$ males, the p-values indicate a bigger difference from the deletion of *Bmal1* than the *Cre* gene itself (41). N-*Bmal1*$^{-/-}$ males have more gonadal fat than N-control males (Figure 3-4). We did not find significant differences between S-*Bmal1*$^{-/-}$, A-*Bmal1*$^{-/-}$, G-*Bmal1*$^{-/-}$ and wild type controls in the size of testis and seminal vesicles.

N-*Bmal1*$^{-/-}$ males display obesity and dwarfism. As shown in Figure 3-5 and 3-6, N-*Bmal1*$^{-/-}$ males had shorter tails than N-control males. At the same time, N-*Bmal1*$^{-/-}$ showed a "pear-

like" body shape. N-*Bmal1*$^{-/-}$ males are heavier than N-control males while the differences are offset by dwarfism (P = 0.053, Wilcoxon rank sum test, Figure 3-7). As a result, the weight/tail ratio is higher in N-*Bmal1*$^{-/-}$ mice than N-controls (P = 0.005, Wilcoxon rank sum test, Figure 3-7). Similar to *Bmal1*$^{-/-}$ males, N-*Bmal1*$^{-/-}$ males displayed a smaller pituitary weight. Furthermore, when we compared the body weight of the fertile N-*Bmal1*$^{-/-}$ males and the infertile N-*Bmal1*$^{-/-}$ males (Table 3-6), we observe that the infertile males weigh more than the fertile males (p = 0.03). In addition, it was observed that the infertile N-*Bmal1*$^{-/-}$ males were not interested in the females, as displayed by lack of smelling or mounting behavior. The S-*Bmal1*$^{-/-}$, A-*Bmal1*$^{-/-}$ and G-*Bmal1*$^{-/-}$ males did not show these defects.

In order to characterize the effect of central nervous system and steroidogenic cell excision of *Bmal1*^{fx} on feeding behavior, we measured the food intake during day and night for 10 continuous days in N-*Bmal1*$^{-/-}$ and *S-Bmal1*$^{-/-}$ mice. Representative pictures of individual mice of indicated genotype were shown in Figure 3-8A. The *Bmal1*$^{fx/fx}$, N-control, S-control and *Bmal1*$^{+/+}$ mice ate more food at night than in day. In contrast, *Bmal1*$^{-/-}$ mice did not display this rhythmic feeding behavior. Overall, N-*Bmal1*$^{-/-}$ mice still ate more food at night than during the day (Figure 3-8b). To document feeding we performed a simple pair-wise comparison of night-day food intake measurements (Figure 3-8c). Since *Bmal1*$^{+/-}$ and *Bmal1*$^{+/+}$ mice did not show differences in feeding behavior, these animals were pooled for analysis of food intake. Similarly, *Baml1*$^{fx/fx}$ and N-controls were pooled to serve as controls for N- *Bmal1*$^{-/-}$, and *Baml1*$^{fx/fx}$ and S-controls were pooled to serve as controls for S- *Bmal1*$^{-/-}$. The night-day food consumption ratio is significantly lower in *Bmal1*$^{-/-}$ mice (n = 5) than *Bmal1*$^{+/\pm}$ mice (n = 5) (P = 2e-10). N-*Bmal1*$^{-/-}$ mice (n = 5) have a smaller ratio than normal control mice (n = 5) (P = 0.0004). S-*Bmal1*$^{-/-}$ mice (n = 5) did not show any difference from normal controls (n = 3).

Serum Testosterone levels in N-*Bmal1*$^{-/-}$ and S-*Bmal1*$^{-/-}$ males. As shown in Figure 3-8, N-*Bmal1*$^{-/-}$ males displayed significantly lower testosterone levels than control normal males. The S-*Bmal1*$^{-/-}$ males all showed a trend of lower testosterone than normal controls (*Bmal1*$^{fx/fx}$, *Bmal1*$^{fx/+}$ and S-control), but the difference is not significant at $P< 0.05$.

***Bmal1*$^{-/-}$ males have "functional" sperm.** *Bmal1*$^{-/-}$ males have histologically normal testis and seminal vesicles (Fig 3-10). Although *Bmal1*$^{-/-}$ males (n = 6) produced ~45% of the sperm count of control *Bmal1*$^{+/+}$ males (n = 6), the motility of the sperm was not significantly different (Table 3-5). In in-vitro fertilization and embryo transfer experiments, the sperm from *Bmal1*$^{-/-}$ males (n = 3) could inseminate and fertilize the oocytes from *Bmal1*$^{+/-}$ (n = 11) and *Bmal1*$^{-/-}$ females (n = 16). Furthermore, when transferred into outbred ICR pseudo-pregnant dams the *Bmal1*$^{-/-}$ embryos developed well, and survived to wean (Table 2-4).

DISCUSSION

One out of 6 couples have problems in conceiving a pregnancy. Unfortunately, in many cases, the underlying cause is elusive. As evidence that disrupted circadian clock could be at play in the biology of reproduction, we note that global *Bmal1*$^{-/-}$ mice are infertile. Unfortunately the broad spectrum of biological functions of *Bmal1/*circadian rhythm in the whole organism makes it difficult to delineate the relative requirements for *Bmal1* in different cell types to fertility. For example, *Bmal1*$^{-/-}$ animals display extensive defects including arthropathy, growth retardation, early aging, diabetes, and disrupted steroidogenesis (40, 42). The defects could result in physical obstacles for copulation behavior, late onset of puberty and low steroid levels which contribute to infertility independently or synergistically.

Male infertility could arise from various defects such as arrested spermatogenesis, abnormal sperm motility or movement, low sperm count, erectile dysfunction, low libido towards females, physical obstacles etc. In our study the failure to copulate might be the main cause of the infertility in *Bmal1*$^{-/-}$ males. Our evidence comes from a number of observations. Firstly, *Bmal1*$^{-/-}$ have normal testicular histological structure and produce functional sperm in vitro. This observation suggests that the testosterone levels in the testis, although not optimal, are high enough for spermatogenesis. Second, we observed a lack of copulation plugs in female partners of *Bmal1*$^{-/-}$ males. Third, global *Bmal1*$^{-/-}$ have ~50% sperm count compared to wild type controls, much higher than the 10% generally considered to result in infertility. Similar to global *Bmal1*$^{-/-}$ males, the infertile N-*Bmal1*$^{-/-}$ males lack copulation behavior displayed by absence of copulation plugs or signs of intromission in their female partners. In summary, defects in male sexual behavior in *Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ males contribute to their subfertility.

Steroid hormones control the display of male sexual behavior. Firstly testosterone is responsible for most aspects of male behavior. Removal of the testes leads to a dramatic reduction in sexual behavior that can be restored by treatment with exogenous testosterone (43). In addition, it is reported that in massively overweight men, serum testosterone decreases as body mass increases (44, 45). We observed lower testosterone levels in central nervous system conditional knockout N-*Bmal1*$^{-/-}$ males. Furthermore, the infertile N-*Bmal1*$^{-/-}$ males weigh more than the fertile N- *Bmal1*$^{-/-}$ males and wild type controls. It is interesting that estrogen is also needed for normal male sexual behavior. The evidence comes from the observance that 50% of Aromatase (the enzyme that converts testosterone to estrogen) knockout males are infertile due to lack of attempt to mount the females (46). It is worth noting that E-box elements are found in the promoter of the aromatase gene and are required for full promoter activity (47, 48). The defects of sexual behavior of *Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ males might be due to defects in steroidogenesis.

The *Bmal1* deletion in the central nervous system could result in low libido through some disruption in neuroendocrine function. N-*Bmal1*$^{-/-}$ mice displayed phenotypes of disrupted neuroendocrinology, such as dwarfism and obesity. In support of this idea, we postulate that the obesity of N-*Bmal1*$^{-/-}$ mice could be an indication of an altered leptin signaling pathway. Serum leptin levels follow circadian rhythms. Moreover, leptin receptors are present in many parts in the central nervous system including SCN neurons. These two pieces of evidence suggests that the leptin signaling system could be under the regulation of circadian clocks. Interestingly the leptin mutant mice *Lep*$^{ob/ob}$ and leptin receptor B mutant mice *LepR*$^{db/db}$ are all obese, hyperphagic and infertile. Dietary restriction could occasionally restore the fertility of *Lep*$^{ob/ob}$ males (49, 50). The dwarfism of N-*Bmal1*$^{-/-}$ mice could be an indication of the affected growth

hormone or PRL signaling pathway. The secretion of growth hormone (GH) and thyrotropin (TSH) follows a 24-hour rhythm (51). Global *Bmal1*$^{-/-}$ mice displayed growth retardation. Both the global *Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ males have shorter tails than wild type controls. These three pieces of evidence suggest that the GH/PRL/TSH system is under circadian control. It is interesting to note that the dwarf mice and the snell dwarf mice with GH/PRL/TSH deficiency are infertile.

The reproductice axis knockout *S-Bmal1*$^{-/-}$and leydig cell knockout A-*Bmal1*$^{-/-}$ males are fertile. S-*Bmal1*$^{-/-}$ and A-*Bmal1*$^{-/-}$ males have normal size of gonads and are not sex-reversed. The normal fertility phenotype of *S-Bmal1*$^{-/-}$ and A-*Bmal1*$^{-/-}$ males suggests that either BMAL1's function in sf-1 expressing cells and Amhr2-expressing cells can be compensated by the function of Bmal1 from other tissues, or the residual steroidogenic functions after Bmal1 deletion in these cells passes the minimum requirement for male fertility. Considering the large amount of sperm it is not hard to imagine that even the sperm count is lower in *Bmal1*$^{-/-}$ males the sperm with good quality in these males can still function as long as copulation occurs.

Males and female mice have different behavioral functions in fertility. Males initiate the mating behavior while females are passive in the process of copulation. The disruption of *Bmal1* in the central nervous system could result in defects in male's mounting behavior. The neural system exhibits highly sexual dimorphic characteristics in many aspects of physiology. Men have bigger SCN than women. There are reports showing the presence of testosterone receptors and estrogen receptors in SCN. There is evidence that testosterone acts during prenatal period to organize the development of aromatase-expressing neurons into the male-typical sexual dimorphic nucleus (SDN). These observations could be underlying the idea that the requirement of an intact central clock for sexual development is different in male and female mice.

Intrauterine fetal position might result in different fetal exposure to steroids. Studies demonstrated that male rats which were gestated between two male fetuses (2M) have 2-fold larger SDN-POA volumes than those gestated between two female fetuses (2F). At the same time, the testosterone levels, as well as the 17β-estradiol (product of testosterone) levels, were found to be significantly larger in 2M males than in 2F males on gestation day 21 (testosterone can be transferred from adjacent male fetuses to the target rats). The fetal position of the embryos could result in variability of phenotype from mice with the same genotype. I hypothesize this could explain 50% of *N-Bmal1*$^{-/-}$ are fertile while the other 50% are infertile.

In contrast to males, female mice are in the passive receiving position in the process of copulation. The female's task, after obtaining sperm from the males, is to make best environment for the survival of the embryos. So the ability of the ovaries to produce progesterone is the key requirement for female fertility. In supporting of this idea, the central nervous system conditional knockout N-*Bmal1*$^{-/-}$ females are fertile, while the peripheral reproductive axis conditional knockout S-*Bmal1*$^{-/-}$ females are infertile. The sensitivity to the requirement for circadian clocks in different tissues to remain reproductive fitness is also different in males and females.

It is worth noting that 2 month old *Cre*^{nestin} mice are affected by hypopituitarism and display low growth hormone, prolactin and thyroid stimulating hormone (41). However, their ACTH and LH levels are unaffected. This suggests that the cells in pit-1 lineage in the pituitary are not functioning well in Nestin/Cre mice. To try to preclude the possibility that compromised fertility of N-*Bmal1*$^{-/-}$ males is a result of the Nestin/Cre itself, we used rGhrhr/cre to delete Bmal1 in cells of the pit-1 lineage. The G- *Bmal1*$^{-/-}$ mice are totally normal, with none of the defects including dwarfism, obesity and infertility we see in N-*Bmal1*$^{-/-}$ mice.

This piece of work is the first report that dissect the roles of *Bmal1* in different tissues to male and female reproductive biology. *Bmal1* deletion in the central nervous system contributes to male infertility while *Bmal1* function in the steroidogenic cells are required to maintain female fertility.

REFERENCES

1. Alvarez JD*, et al.* (2008) The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J Biol Rhythms* 23(1):26-36.
2. Ratajczak CK, Boehle KL, & Muglia LJ (2009) Impaired steroidogenesis and implantation failure in Bmal1-/- mice. *Endocrinology* 150(4):1879-1885.
3. Anderson K, Nisenblat V, & Norman R (2010) Lifestyle factors in people seeking infertility treatment - A review. *Aust N Z J Obstet Gynaecol* 50(1):8-20.
4. Labyak S, Lava S, Turek F, & Zee P (2002) Effects of shiftwork on sleep and menstrual function in nurses. *Health Care Women Int* 23(6-7):703-714.
5. Guilding C & Piggins HD (2007) Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain? *Eur J Neurosci* 25(11):3195-3216.
6. Tischkau SA, Howell RE, Hickok JR, Krager SL, & Bahr JM (2011) The luteinizing hormone surge regulates circadian clock gene expression in the chicken ovary. *Chronobiol Int* 28(1):10-20.
7. Sellix MT & Menaker M (2011) Circadian clocks in mammalian reproductive physiology: effects of the "other" biological clock on fertility. *Discov Med* 11(59):273-281.
8. Stutz AM, Staszkiewicz J, Ptitsyn A, & Argyropoulos G (2007) Circadian expression of genes regulating food intake. *Obesity (Silver Spring)* 15(3):607-615.
9. Lowrey PL & Takahashi JS (2004) Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet* 5:407-441.
10. Van der Beek EM, Horvath TL, Wiegant VM, Van den Hurk R, & Buijs RM (1997) Evidence for a direct neuronal pathway from the suprachiasmatic nucleus to the gonadotropin-releasing hormone system: combined tracing and light and electron microscopic immunocytochemical studies. *J Comp Neurol* 384(4):569-579.
11. Wiegand SJ & Terasawa E (1982) Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. *Neuroendocrinology* 34(6):395-404.
12. Hickok JR & Tischkau SA (2010) In vivo circadian rhythms in gonadotropin-releasing hormone neurons. *Neuroendocrinology* 91(1):110-120.
13. Chappell PE, White RS, & Mellon PL (2003) Circadian gene expression regulates pulsatile gonadotropin-releasing hormone (GnRH) secretory patterns in the hypothalamic GnRH-secreting GT1-7 cell line. *J Neurosci* 23(35):11202-11213.
14. Yoon H, Enquist LW, & Dulac C (2005) Olfactory inputs to hypothalamic neurons controlling reproduction and fertility. *Cell* 123(4):669-682.
15. Kalra SP & Kalra PS (1996) Nutritional infertility: the role of the interconnected hypothalamic neuropeptide Y-galanin-opioid network. *Front Neuroendocrinol* 17(4):371-401.
16. Chao PT, Yang L, Aja S, Moran TH, & Bi S (2011) Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity. *Cell Metab* 13(5):573-583.
17. Gamble KL, Ehlen JC, & Albers HE (2005) Circadian control during the day and night: Role of neuropeptide Y Y5 receptors in the suprachiasmatic nucleus. *Brain Res Bull* 65(6):513-519.
18. Luquet S, Perez FA, Hnasko TS, & Palmiter RD (2005) NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310(5748):683-685.
19. Dyzma M, Boudjeltia KZ, Faraut B, & Kerkhofs M (2010) Neuropeptide Y and sleep. *Sleep Med Rev* 14(3):161-165.

20. Glass JD, Guinn J, Kaur G, & Francl JM (2010) On the intrinsic regulation of neuropeptide Y release in the mammalian suprachiasmatic nucleus circadian clock. *Eur J Neurosci* 31(6):1117-1126.
21. Stütz AM, Staszkiewicz J, Ptitsyn A, & Argyropoulos G (2007) Circadian expression of genes regulating food intake. *Obesity (Silver Spring)* 15(3):607-615.
22. Leclerc GM & Boockfor FR (2005) Pulses of prolactin promoter activity depend on a noncanonical E-box that can bind the circadian proteins CLOCK and BMAL1. *Endocrinology* 146(6):2782-2790.
23. Resuehr D, Wildemann U, Sikes H, & Olcese J (2007) E-box regulation of gonadotropin-releasing hormone (GnRH) receptor expression in immortalized gonadotrope cells. *Mol Cell Endocrinol* 278(1-2):36-43.
24. Son GH*, et al.* (2008) Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci U S A* 105(52):20970-20975.
25. Sadacca LA, Lamia KA, deLemos AS, Blum B, & Weitz CJ (2011) An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice. *Diabetologia* 54(1):120-124.
26. Sukumaran S, Xue B, Jusko WJ, Dubois DC, & Almon RR (2010) Circadian variations in gene expression in rat abdominal adipose tissue and relationship to physiology. *Physiol Genomics* 42A(2):141-152.
27. Morse D, Cermakian N, Brancorsini S, Parvinen M, & Sassone-Corsi P (2003) No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. *Mol Endocrinol* 17(1):141-151.
28. Lefta M, Wolff G, & Esser KA (Circadian rhythms, the molecular clock, and skeletal muscle. *Curr Top Dev Biol* 96:231-271.
29. Bingham NC, Verma-Kurvari S, Parada LF, & Parker KL (2006) Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44(9):419-424.
30. Dubois NC, Hofmann D, Kaloulis K, Bishop JM, & Trumpp A (2006) Nestin-Cre transgenic mouse line Nes-Cre1 mediates highly efficient Cre/loxP mediated recombination in the nervous system, kidney, and somite-derived tissues. *Genesis* 44(8):355-360.
31. Piper ML, Unger EK, Myers MG, & Xu AW (2008) Specific physiological roles for signal transducer and activator of transcription 3 in leptin receptor-expressing neurons. *Mol Endocrinol* 22(3):751-759.
32. Yin Z, Williams-Simons L, Rawahneh L, Asa S, & Kirschner LS (2008) Development of a pituitary-specific cre line targeted to the Pit-1 lineage. *Genesis* 46(1):37-42.
33. Xu Q*, et al.* (2007) Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells. *Endocrine* 32(1):96-106.
34. Pelusi C, Ikeda Y, Zubair M, & Parker KL (2008) Impaired follicle development and infertility in female mice lacking steroidogenic factor 1 in ovarian granulosa cells. *Biol Reprod* 79(6):1074-1083.
35. Wang Y*, et al.* (2005) Increases in phosphorylation of SAPK/JNK and p38MAPK correlate negatively with mouse embryo development after culture in different media. *Fertil Steril* 83 Suppl 1:1144-1154.
36. Freking BA, Purdy PH, Spiller SF, Welsh CS, & Blackburn HD (2012) Boar sperm quality in lines of pigs selected for either ovulation rate or uterine capacity. *J Anim Sci*.
37. Tronche F*, et al.* (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23(1):99-103.

38. Kim KW*, et al.* (2010) CNS-specific ablation of steroidogenic factor 1 results in impaired female reproductive function. *Mol Endocrinol* 24(6):1240-1250.
39. Kim KW, Zhao L, & Parker KL (2009) Central nervous system-specific knockout of steroidogenic factor 1. *Mol Cell Endocrinol* 300(1-2):132-136.
40. Bunger MK*, et al.* (2005) Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis* 41(3):122-132.
41. Galichet C, Lovell-Badge R, & Rizzoti K (2010) Nestin-Cre mice are affected by hypopituitarism, which is not due to significant activity of the transgene in the pituitary gland. *PLoS One* 5(7):e11443.
42. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, & Antoch MP (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes Dev* 20(14):1868-1873.
43. Hull EM & Dominguez JM (2007) Sexual behavior in male rodents. *Horm Behav* 52(1):45-55.
44. Cervero A*, et al.* (2006) The role of the leptin in reproduction. *Curr Opin Obstet Gynecol* 18(3):297-303.
45. Betancourt-Albrecht M & Cunningham GR (2003) Hypogonadism and diabetes. *Int J Impot Res* 15 Suppl 4:S14-20.
46. Robertson KM, Simpson ER, Lacham-Kaplan O, & Jones ME (2001) Characterization of the fertility of male aromatase knockout mice. *J Androl* 22(5):825-830.
47. Furbass R, Said HM, Schwerin M, & Vanselow J (2001) Chromatin structure of the bovine Cyp19 promoter 1.1. DNaseI hypersensitive sites and DNA hypomethylation correlate with placental expression. *Eur J Biochem* 268(5):1222-1227.
48. Furbass R, Tomek W, & Vanselow J (2010) Upstream stimulating factors 1 and 2 enhance transcription from the placenta-specific promoter 1.1 of the bovine cyp19 gene. *BMC Mol Biol* 11:5.
49. DICKIE PWLaMM (1954) *Relative Sterility in Obese Males* Corrected by Dietary Restriction*Journal of heridity* 45:56-58.
50. Chehab FF, Lim ME, & Lu R (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet* 12(3):318-320.
51. Behrends J, Prank K, Dogu E, & Brabant G (1998) Central nervous system control of thyrotropin secretion during sleep and wakefulness. *Horm Res* 49(3-4):173-177.

FIGURE LEGENDS

Figure 3-1. tissue-specific excision of the conditional *Bmal1*^{fx} allele.

In S-*Bmal1*$^{-/-}$ females the *Bmal1*^{fx}-excised allele is detected in the brain, pituitary, adrenal gland, and ovaries and is absent in liver, muscle, uterus and oviduct. In S-*Bmal1*$^{-/-}$ males, the *Bmal1*^{fx}-excised allele is detected in the brain, pituitary, adrenal gland, and testis and is absent in liver, muscle and seminal vesicles. In N-*Bmal1*$^{-/-}$ females the *Bmal1*^{fx}-excised allele is detected in the brain, pituitary, muscles, and ovaries and is absent in liver, adrenal, uterus and oviduct. In N-*Bmal1*$^{-/-}$ males the *Bmal1*^{fx}-excised allele is detected in the brain, pituitary, muscles, and testis, and absent in liver, adrenal, and seminal vesicles.

Figure 3-2. N-*Bmal1*$^{-/-}$ and S-*Bmal1*$^{-/-}$ mice are absent from arthropathy. (A) N-*Bmal1*$^{-/-}$ and S-*Bmal1*$^{-/-}$ mice don't show arthropathy at 180 days. Alizarin Red–stained hind limbs showed that N-*Bmal1*$^{-/-}$ and S-*Bmal1*$^{-/-}$ mice don't show tendon calcification (arrow) that is present in *Bmal1*$^{-/-}$ mice. B. N-*Bmal1*$^{-/-}$ and S-*Bmal1*$^{-/-}$ males displayed similar body weight with wild type at ~220 days.

Figure 3-3. Effect of *Bmal1* deletion in nestin-expressing cells on the estrous cycle. A, Representative patterns of estrous cycles from mutants (upper 2 panels, N-*Bmal1*$^{-/-}$ females) and their littermate control (botomn panel Bmal1$^{fx/fx}$ and N-control). Each day is represented by a diamond. E=estrus, P= proestrus, D= diestrus. B, consecutive days in estrus. Each dot represents an event of "y" number of consecutive days in estrus.

Figure 3-4. Effect of *Bmal1* deletion in sizes of seminal vesicles (a), testis (b) and gonadal fat (c). *Bmal1*$^{+/-}$, N-control males displayed smaller seminal vesicles and testis while bigger gonadal fat than *Bmal1*$^{-/-}$ and N-*Bmal1*$^{-/-}$ males, Wilcox rank sum test.

Figure 3-5. representative pictures of conditional knockout *Bmal1* mutants. seminal vesicle (A), body shape (B,C) and size of pituitary (D).

Figure 3-6. Effect of *Bmal1* deletion in length of tail (a) and size of pituitary (b). *Bmal1*$^{+/-}$ males displayed shorter tails and smaller pituitaries than *Bmal1*$^{-/-}$ males. N-control displayed shorter tail than N-*Bmal1*$^{-/-}$ males. P-value by Wilcox rank sum test.

Figure 3-7. Effect of *Bmal1*deletion in body weight and body weight/tail length ratio.

Figure 3-8. Effect of *Bmal1* deletion on feeding behavior. Represenattive pictures of individual mice of indicated genotype were shown in (a). Food consumption at night (Δ) and in day (◊) were shown for 10 continuous days. (b) Overall average food intake at night and in day were shown in columns. (c) Simple pair-wise comparison of night/day food intake measurements . Each dot represents one day of a mouse. P-value by Wilcoxon rank sum test.

Figure 3-9. Effect of *Bmal1* deletion on serum testosterone.

Figure 3-10. Effect of *Bmal1* deletion on testis. Representative pictures from *Bmal1*$^{-/-}$ and *Bmal1*$^{+/+}$. Scale bar, 250um.

TABLE LEGENDS

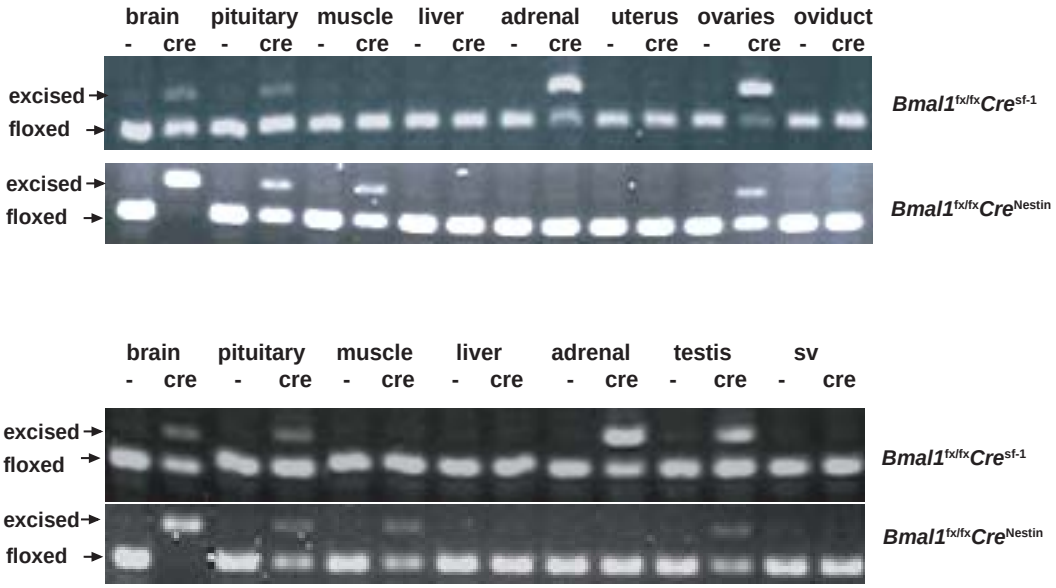
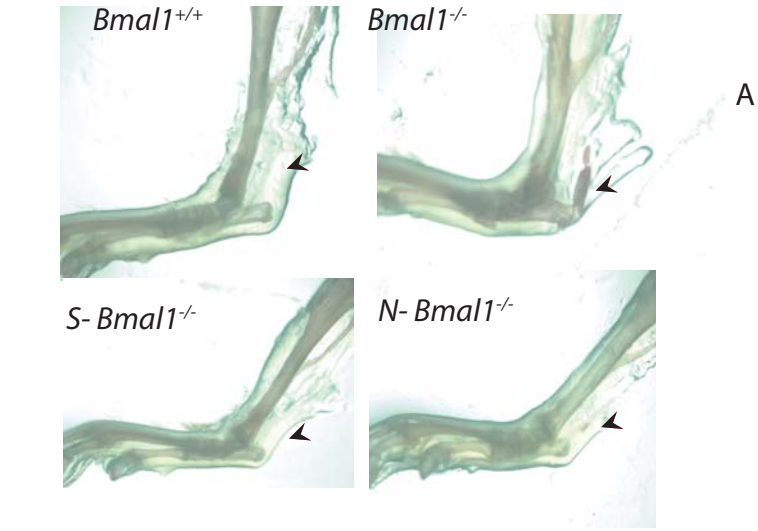
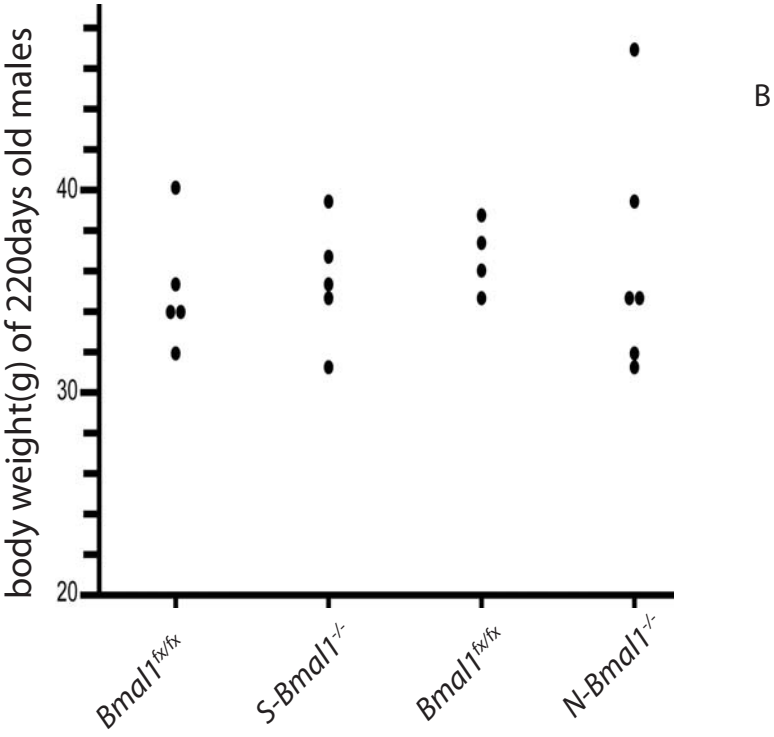


Figure 3-1

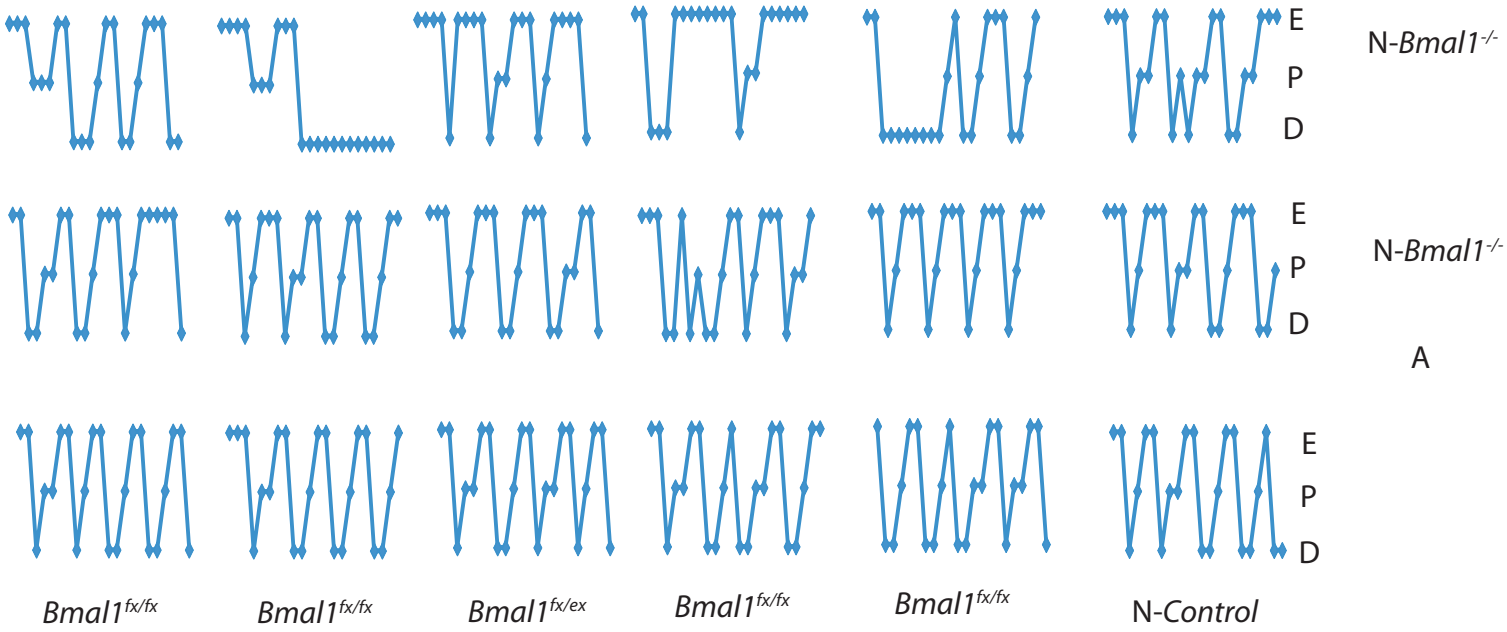


A



B

Figure 3-2



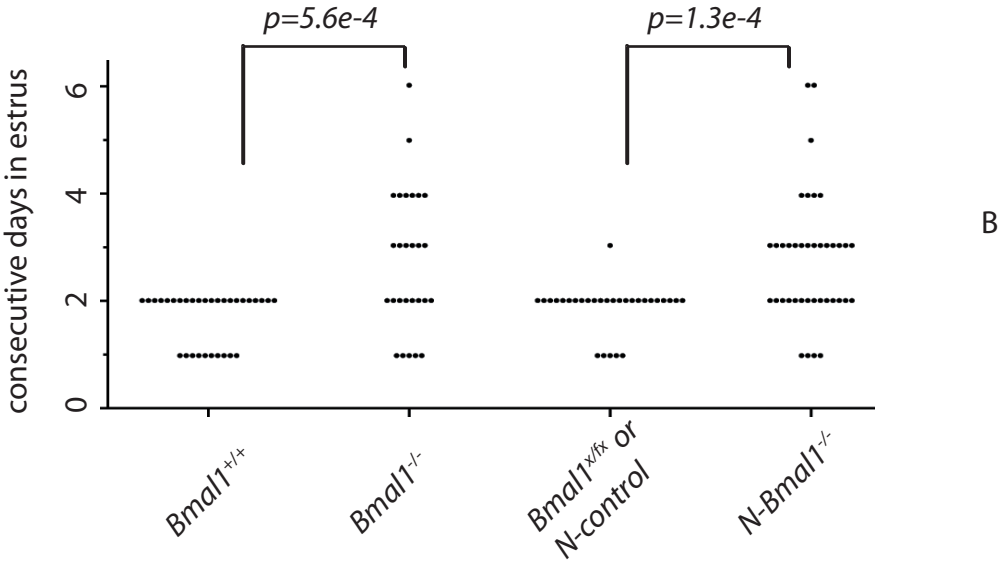


Figure 3-3

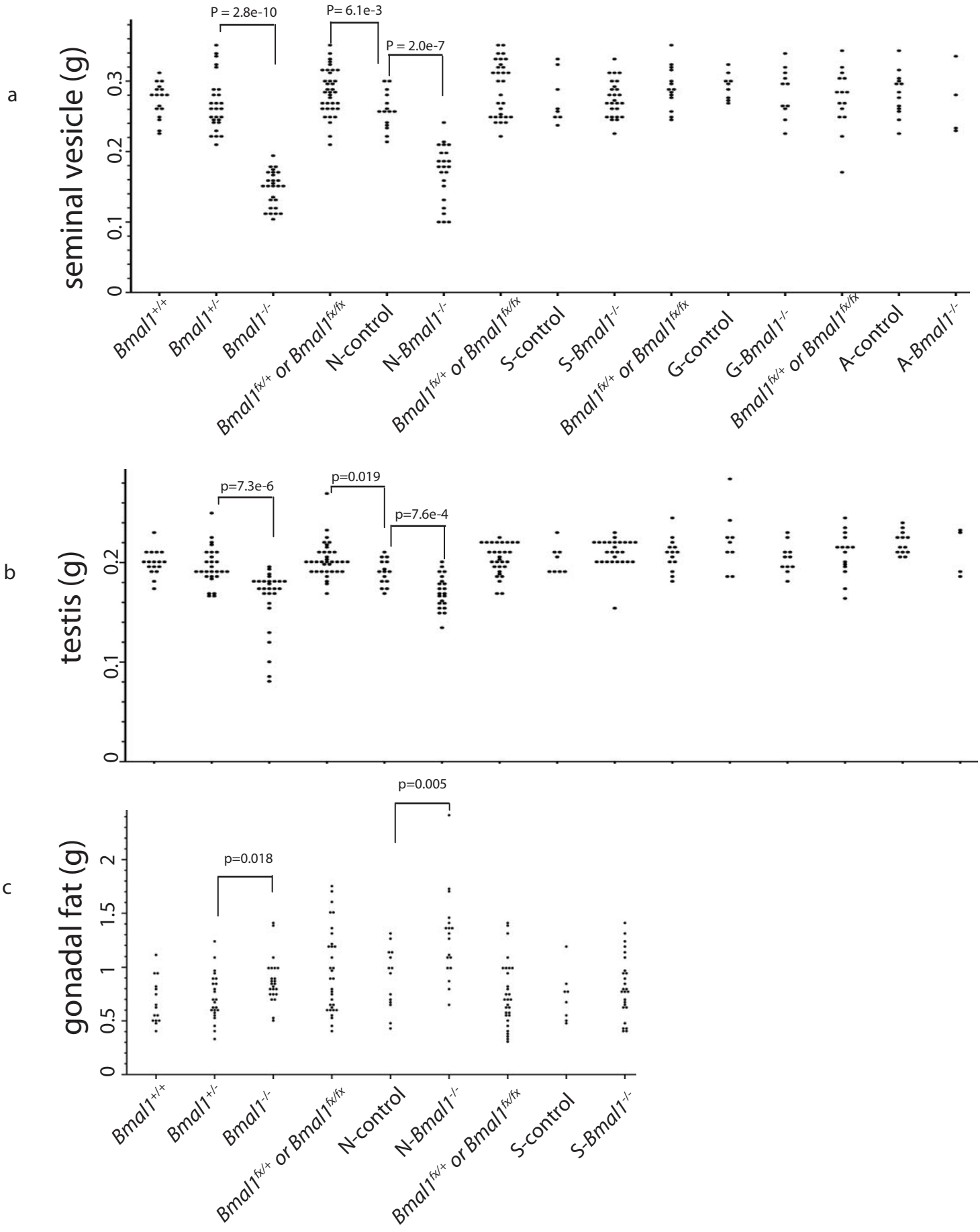
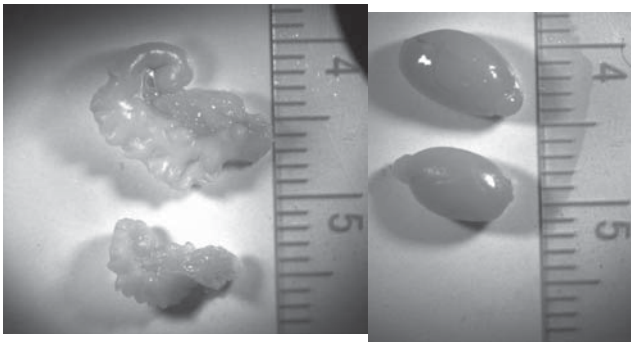


Figure 3-4

N-control

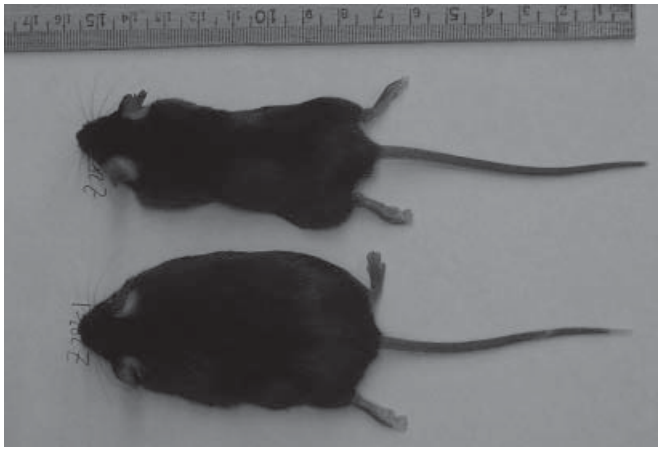
N-*Bmal1*$^{-/-}$



A

Bmal1$^{fx/fx}$

Infertile
N-*Bmal1*$^{-/-}$

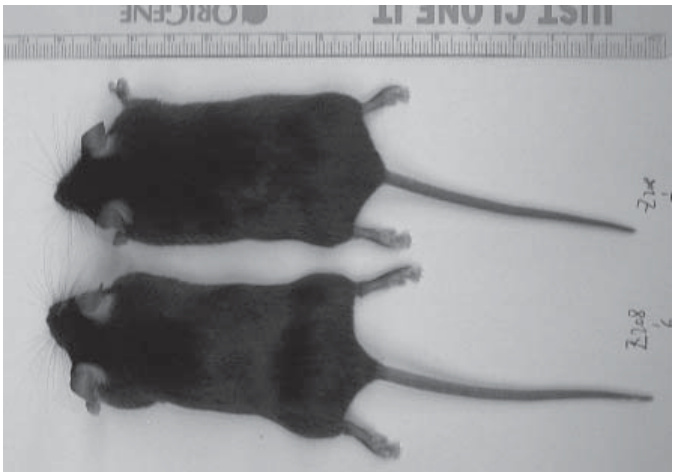


B

Figure 3-5

N-*Bmal1*$^{-/-}$

Bmal1$^{fx/fx}$



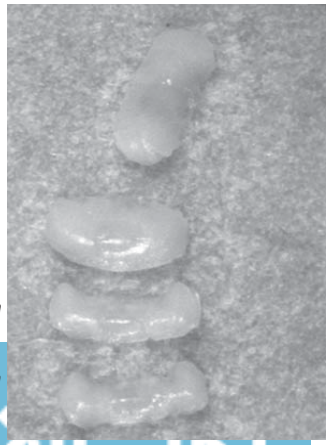
c

Bmal1$^{fx/+}$

Bmal1$^{fx/ex}$

Bmal1$^{fx/fx}$*Cre*^{nestin}

Bmal1$^{fx/ex}$*Cre*^{nestin}



D

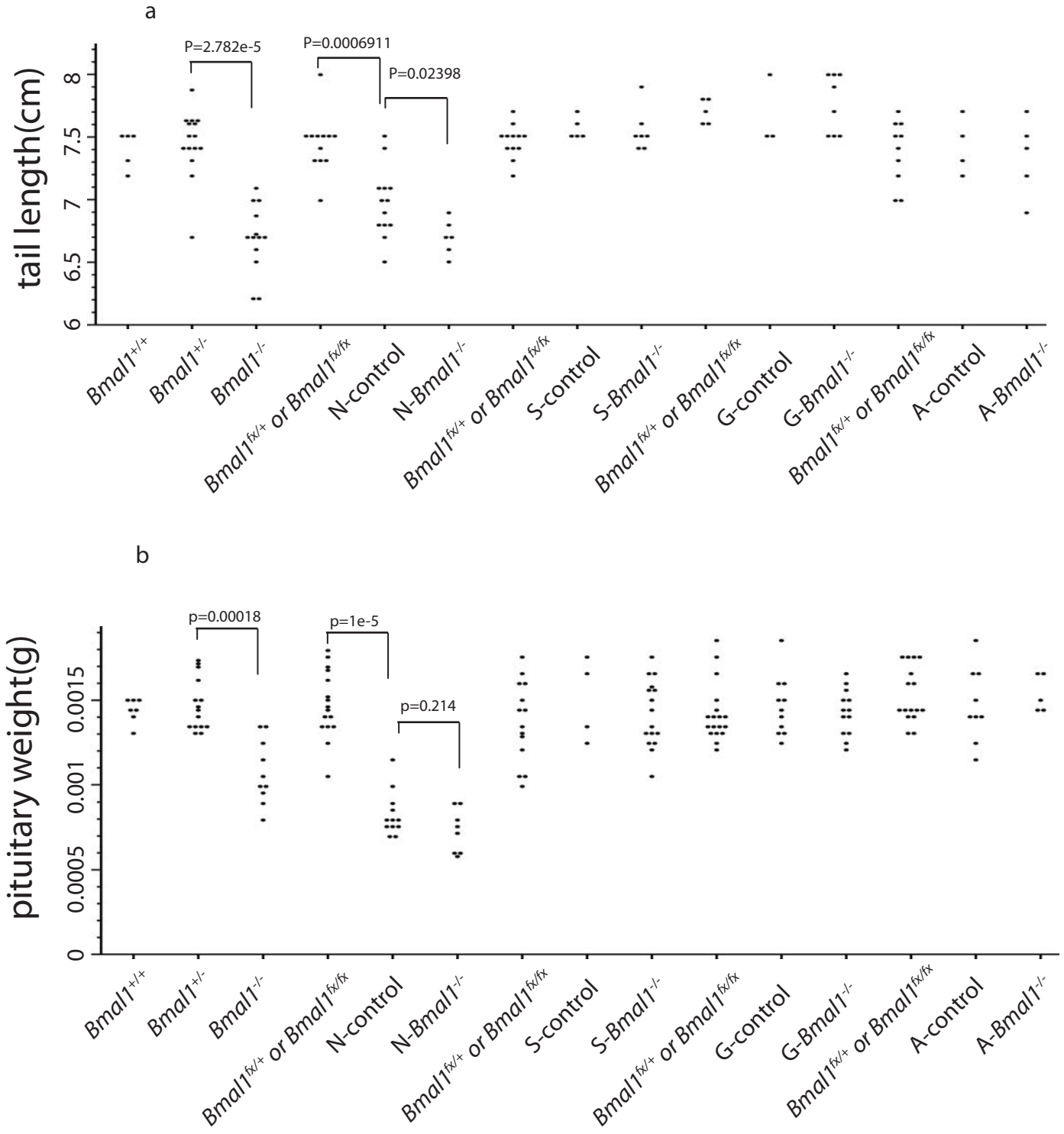


Figure 3-6

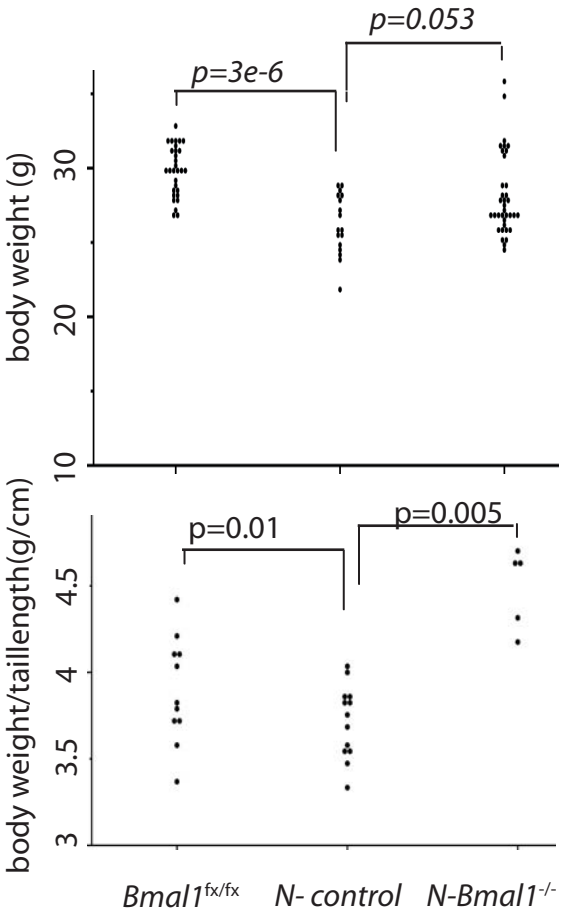


Figure 3-7

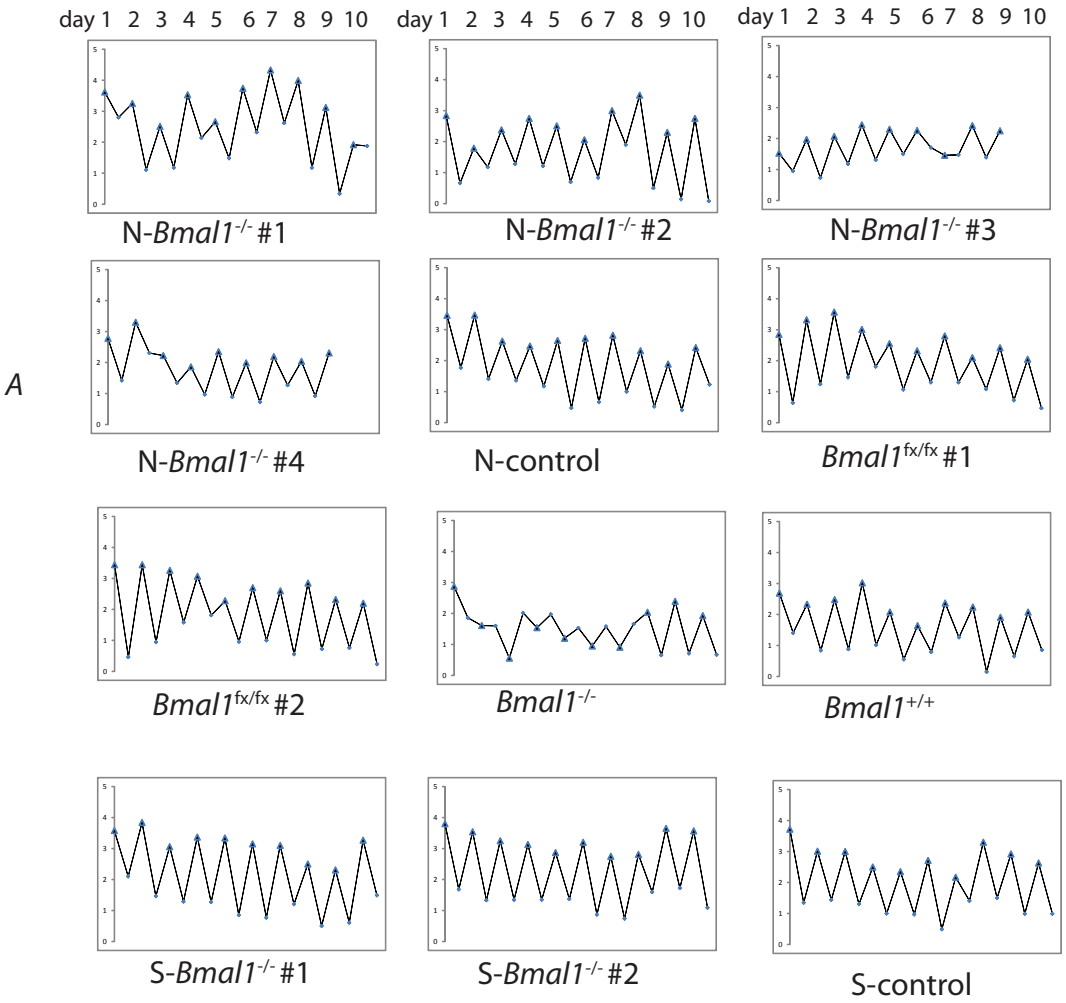
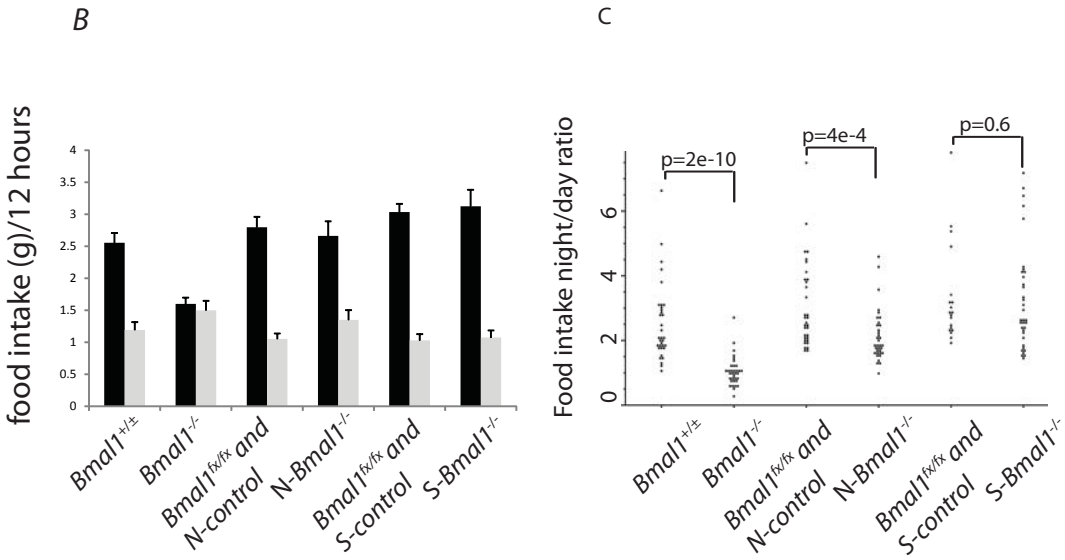


Fig 3-8



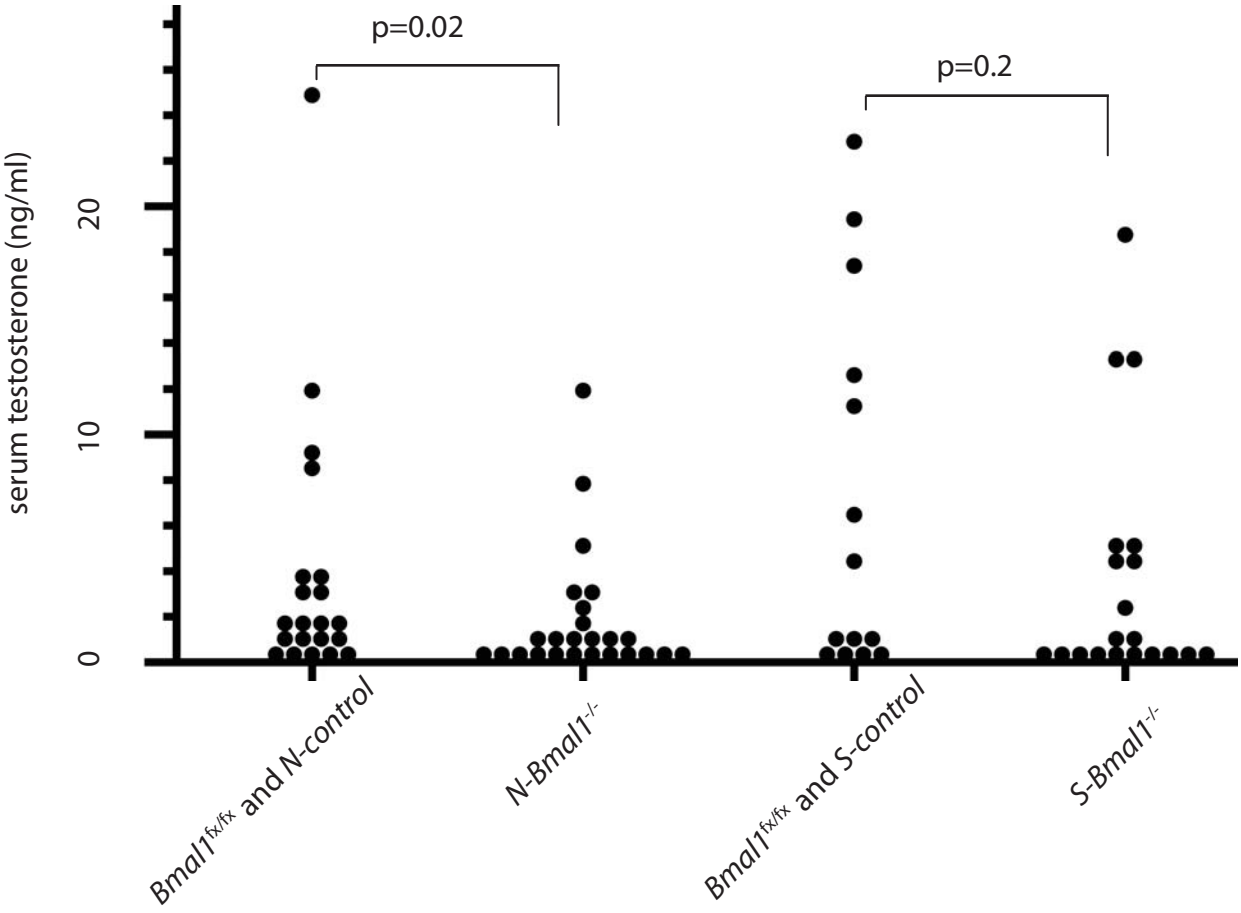
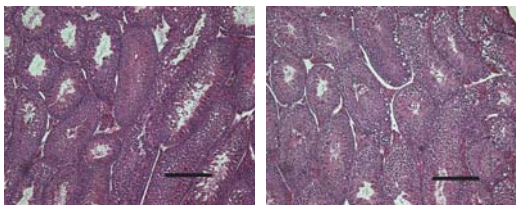


Figure 3-9



Bmal1$^{-/-}$ *Bmal1*$^{+/+}$

Figure 3-10

Table 3-1 Cre lines used and the targeting cell types

Cre line	Targeted tissues
B6.Cg-Tg(Nes-cre)1Kln/J	central and peripheral nervous system with a few isolated kidney and heart cells
Sf-1/cre	the somatic cells of the gonads, the adrenal cortex, the anterior pituitary, the spleen, the VMH nucleus
Amhr2/cre	Müllerian duct, granulosa cells, leydig cells
rGhrhr/Cre	the Pit1 lineage, including somatotrophs, lactotrophs, and thyrotrophs

Table3-2 effect of *Bmal1* deletion on male fertility

Male genotype	No. of males tested	No. of males that are fertile	% of fertility
Bmal1$^{+/-}$	10	10	100
Bmal1$^{-/-}$	20	1	5[a]
Bmal1$^{fx/fx}$	17	17	100
N-control	9	9	100
N-*Bmal1*$^{-/-}$	17	8	47[b,c]
S-*Bmal*$^{-/-}$	17	17	100
A-*Bmal*$^{-/-}$	6	6	100
G- *Bmal*$^{-/-}$	5	5	100

a, p-value<0.001 *Bmal1*$^{-/-}$ vs *Bmal1*$^{+/-}$ by chi-square test

b, P-value<0.05 N-*Bmal1*$^{-/-}$ vs N-control by chi-square test

c, One N-*Bmal1*$^{-/-}$ male mouse, which sired a litter 114 days after mating, is considered as infertile because the parturition happened beyond our inspection window(50days).

Table 3-3 effect of *Bmal1* deletion on female fertility

Female genotype	No. of females tested	No. of females that are fertile	% of fertility
Bmal1$^{fx/fx}$	8	8	100
N-control	14	14	100
N-*Bmal1*$^{-/-}$	19	16	85
S-control	19	19	100
S-*Bmal*$^{-/-}$	12	0	0[a]
A-control	12	11	92
A-*Bmal*$^{-/-}$	20	14	70
G-control	5	5	100
G- *Bmal*$^{-/-}$	10	10	100

a, p-value<0.001 S-*Bmal1*$^{-/-}$ vs S-control, chi-square test

Table 3-4 effect of *Bmal1* deletion on male organ measurements

male organ measurements									
	Bmal1$^{+/+}$ (n=17)	*Bmal1*$^{+/-}$ (n=27)	*Bmal1*$^{-/-}$ (n=27)	*Bmal1*$^{+/fx}$ or *Bmal1*$^{fx/fx}$ (n=36)	*N-control* (n=15)	*N-Bmal1*$^{-/-}$ (n=23)	*Bmal1*$^{+/fx}$ or *Bmal1*$^{fx/fx}$ (n=30)	*S-control* (n=8)	*S-Bmal1*$^{-/-}$ (n=26)
Seminal vesicles(g)	0.273±.006	0.267±.007 P=2.8e-10	0.147±.005	0.285±.005 P=6.1e-3	0.258±.007	0.169±.009 P=2e-7	0.29±.007	0.275±.012	.29±.005
Testis(g)	0.199±.003	0.196±0.004 P=7.3e-6	0.164±.006	0.202±.003 P=0.019	0.19±.003	0.17±.004 P=7.6e-4	0.203±.003	0.202±.005	0.21±.003
Gonadal fat(g)	0.68±.05	0.72±.05 P=0.018	0.87±.04	0.95±.07	0.9±.07 P=0.005	1.27±.09	0.74±.06	0.72±.08	0.82±.06

male organ measurements						
	Bmal1$^{+/fx}$ or *Bmal1*$^{fx/fx}$ (n=14)	*G-control* (n=9)	*G-Bmal1*$^{-/-}$ (n=11)	*Bmal1*$^{+/fx}$ or *Bmal1*$^{fx/fx}$ (n=15)	*A-control* (n=13)	*A-Bmal1*$^{-/-}$ (n=4)
Seminal vesicles(g)	0.29±.008	0.293±.006	0.284±.01	0.275±.011	0.282±.009	0.27±.024
Testis(g)	0.21±.01	0.22±.01	0.204±.004	0.209±.006	0.219±.003	0.209±.012

Table 3-5 effect of Bmal1 deletion on semen

	Bmal1$^{-/-}$	*Bmal1*$^{+/+}$
Sperm count(x10^6/ml) ±SEM	6.8±1[a]	14.8± 3 [a] (p=0.03)
% Total Motile ±SEM	48.2±4.1	60.6±3.9

a Wilcoxon rank sum test. n=6 for each genotype.

Table 3-6. effect of body weight and *Bmal1* deletion on fertility

Age (days)	N-control (g)	Fertile N-*Bmal1*$^{-/-}$ (g)	Infertile N-*Bmal1*$^{-/-}$ (g)
155		26.3	
155		27	40
192	31.3		44.6
220	33	31.8	34.9
220			34.9
220		39.4	40.4
230	34	41	46.9
365	35.33	39.76	43.64

a, p=0.4 between N-control and fertile N-*Bmal1*$^{-/-}$ mice by Wilcoxon rank sum test(one-sided)

b, p=0.03 between fertile N-*Bmal1*$^{-/-}$ and infertile N-*Bmal1*$^{-/-}$ females by Wilcoxon rank sum test(one-sided)

CHAPTER IV

Future directions

INTRODUCTION

This thesis work was designed to our understanding of *Bmal1*, the core circadian clock component, and its role in murine reproductive biology. In the process, this work opened up several new windows for further study. In this chapter, we propose approaches to elucidate additional questions that arose from this thesis, and further discuss some of the lessons learned.

CIRCADIAN CLOCKS AND REPRODUCTIVE BIOLOGY

CamkII/Cre, GnRH/Cre, LepR/Cre Although $Bmal1^{fx/fx}Cre^{nestin}$ males have shown the importance of clocks in central nervous system for male fertility, elucidating the contributions of specific neurons requires more neuron-specific knockout models. We propose to study the reproductive phenotype of male mice by introducing CamkII/Cre, GnRH/Cre, LepR/Cre mice.

CamkII/Cre is expressed around birth in almost all forebrain neurons and can serve as a substitute for Nestin/Cre (1) (2). The Nestin/Cre mice used in the thesis are reported to have hypopituitarism (3). Although we alternatively circumvent this deficit by using hemizygous $Bmal1^{fx/+}Cre^{nestin}$ (N-control) as controls, an alternative central nervous knockout system could give us higher confidence on the role of clocks in central nervous system in male reproductive biology.

Therefor, we propose use of $Bmal1^{fx/fx}Cre^{GnRH}$ in the future to permit specific deletion of circadian clocks in GnRH neurons of male mice (1). Although there are reports that *Bmal1* is expressed in GnRH neurons (4), together with the tremendous studies showing that circadian clocks regulate GnRH release through neuropeptides and neural projections (5-11), the detailed picture of the geographic distribution of *GnRH* neurons and *Bmal1*-expressing cells in the brain is still not known. In addition, although there is this big hypothesis that the SCN and GnRH

controls the diurnal rhythms of LH and FSH and thus reproductive cycle, there is also report arguing against this model (12). Use of central nervous system Cre lines *Cre*^{nestin} gave us some clues on these questions by deleting *Bmal1* in GnRH neurons. In my study the *Bmal1*$^{fx/fx}$*Cre*^{nestin} females are fertile and *Bmal1*$^{fx/fx}$*Cre*^{nestin} males are subfertile, suggesting a sex-dimorphic role of clocks in the central nervous system. However, although Cre^{nestin} has been widely used to serve as a central nervous delete Cre mouse line, to what extent *Cre*^{nestin} deletes target gene on GnRH neurons is not known. The observation of only 10% GnRH neurons could still support maturation and fertility in female mice make the interpretation even more complicated (13). Use of GnRH-specific Cre lines will help us clarify the questions circadian clocks directly regulate GnRH neurons and whether the GnRH neuron-specific knock out result in sex-dimorphic disruption of reproductive outcomes in males and females.

Similarly use of Cre^{LepR} could permit specific and thorough deletion in *lepR*-expressing cells (14). The deletion of *Bmal1* in *LepR*-expressing cells (*Bmal1*$^{fx/fx}$*Cre*^{lepR}) are hopothesized to result in obesity and compromised male fertility. Leptin is an important regulator of energy balance and represents the permissive metabolic signals for reproduction (15). Although leptin levels and hypothalamic gene expression of leptin receptor follow circadian rhythms and there are putative E-box elements in the promoter of leptin gene (16), it is unclear whether these observations reflect direct molecular clock control of the leptin/leptin receptor signalling system. In our study the conditional central nervous system *Bmal1* knockout (*Bmal1*$^{fx/fx}$*Cre*^{nestin}) males display an obesity phenotype, suggesting a direct link between clocks in the central nervous system and obesity, and thus fertility. But the penetrance of infertility and obesity is only 50%. This could be an effect of unstable Cre^{nestin} line (17) or from the incomplete overlap between nestin-expressing cells and lepR-expressing cells in the nervous system. The use of

Bmal1$^{fx/fx}$*Cre*^{lepR} mice will delete circadian clocks in the lepR-expressing cells and thus create a more specific and though mouse model to study the relationship of circadian clocks and obesity and even reproductive biology.

Comparison of the phenotype of *Bmal1*$^{fx/fx}$*Cre*^{CamKII}, *Bmal1*$^{fx/fx}$*Cre*^{GnRH} and *Bmal1*$^{fx/fx}$*Cre*^{lepR} mice will give finer mapping of function or ramification of clock on different neurons and thus tell us the direct or indirect roles of *Bmal1* on obesity and fertility. This study is even more interesting when it turns out that the SCN-specific deletion of *Bmal1* (*Bmal1*$^{fx/fx}$Cre^{syt10}) mice don't display obsesity or infertility phenotype although the the locomotor rhythms are altered (personal communications with Dr Eichele and (18)), suggesting a more complicated interaction system exist among circadian rhythm, obesity and fertility.

***Y4*$^{-/-}$*Bmal1*$^{-/-}$** The double mutant *Y4*$^{-/-}$*Bmal1*$^{-/-}$ male mice are hypothesized to be fertile and lean. Hypothalamic neuropeptide Y (NPY) is a neuromodulator implicated in the control of energy balance (19) and is overproduced in the hypothalamus of *ob*/*ob* mice (20). NPY Y4 receptor knockout rescues fertility in *ob/ob* mice (21). Elevated NPY expression inhibits reproductive function through suppression of GnRH secretion. NPY immunoreactive axons exist in the hypothalamus including SCN (Figure 1-6). The application of NPY to the SCN caused phase shift in the rhythm (22). *Bmal1and NPY* expression follows 24 hour rhythm in hypothalamic clonal mHypoE-44 neurons (23). Plenty of studies established linkage between NPY, obesity, circadian rhythm and fertility (20, 24-28). I propose to generate double mutants of NPY receptor 4 and Bmal1 (*Y4*$^{-/-}$*Bmal1*$^{-/-}$**).** If *Y4*$^{-/-}$*Bmal1*$^{-/-}$male mice are fertile and lean, a definitive pathway involving NPY in circadian control of fertility can be established.

***Bmal1* disruption and *StAR* expression**

StAR directly regulate steroidogenesis and thus may contribute to the reduced testosterone levels in *Bmal1* mutant males. There is direct binding of CLOCK:BMAL1 to the E-box elements in promoter of *StAR* genes (29). We detected reduced testosterone levels in central nervous system *Bmal1* knockout males ($Bmal1^{fx/fx}Cre^{nestin}$). Whether this is an effect of local reduced *StAR* expression is not known yet. We propose *studying StAR* expression in the testis of $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ and other mutants by doing in situ hybridization or QT-PCR.

***Bmal1* disruption and *Avp, Vip* expression**

In my study conditional deletion of *Bmal1* in reproductive axis($Bmal1^{fx/fx}Cre^{sf\text{-}1}$) and the steroidogenic leydig cells ($Bmal1^{fx/fx}Cre^{Amhr2}$) did not result in male infertility. This is arguing against the previous model generated by Alvarez (30). In that model, the reduced expression of testis *StAR* gene is resulted from a loss of intrinsic testis clock, in which BMAL1:CLOCK directly transactivate StAR expression by binding to the E-box elements in its promoter. This defects results in reduced testosterone and thus infertility in global $Bmal1^{-/-}$ male mice.

I hypothesize that in $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ and $Bmal1^{fx/fx}Cre^{Amhr2}$ males normal expression of AVP and VIP can compensate for the effect of local loss of *Bmal1* in testis, and thus AVP and VIP signalling contribute to male fertility in mice. AVP and VIP are neural peptide hormones that modulate a variety of physiology around the body. This hypothesis is based on the following observance. First, $Bmal1^{fx/fx}Cre^{sf\text{-}1}$ males have normal feeding rhythms, suggesting intact SCN master clocks. In addition, sf-1 expression is not detected in SCN (personal communication between Dr. Parker and (31). Second, AVP follows robust circadian rhythm and there are E-box elements in the promoter of *Avp* gene. VIP immunoactive axons exist in SCN (32). Finally, rodent models with *Avp* and *Vip* mutations display defects in male reproductive biology (33)

(34). To test this hypothesis I propose to study the AVP and VIP levels by in situ hybridization or Q-PCR in the SCN of *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ and *Bmal1*$^{fx/fx}$*Cre*^{Amhr2} males first. *Bmal1*$^{fx/fx}$*Cre*$^{sf-1}$ and *Bmal1*$^{fx/fx}$*Cre*^{Amhr2} males are hypothesized to display normal levels of AVP and VIP.

Prenatal treatment with steroids

Experimental studies in animals indicate that androgen exposure in fetal or neonatal life largely accounts for known sex differences in brain structure and behavior (35).

In our study the central nervous system conditional *Bmal1* knockout males could be divided into two groups: the fertile group and the infertile group, with the infertile group always more obese than the fertile group.

I hypothesize the deficiency of steroid exposure in prenatal or neonatal life contributes to the 50% of subfertility in the central nervous system *Bmal1* conditional knockout (*Bmal1*$^{fx/fx}$*Cre*^{nestin}) male mice. These hypotheses are based on the following observation. First, global *Bmal1*$^{-/-}$ and infertile *Bmal1*$^{fx/fx}$*Cre*^{nestin} males are infertile most likely due to behaviral problems. The absence of copulation plugs in their female partners indicate defects in their sexual behavior. Second, global *Bmal1*$^{-/-}$ males have functional sperm that can fertilize oocytes in vitro. The histological sections confirm normal spermatogenesis even in global deletion of *Bmal1*. Thirdly, prenatal and adult exposure to steroids can alter circadian activity patterns in mice evidenced by significant different circadian activity parameters in Aromatase knockout mice compared to wild type mice (36). Aromatase converts androgen to estrogen. Interestingly although with good size of testis and elevated levels of serum testosterone, Aromatase knockout male mice is subfertile due to impaired mounting behavior (37). There is E-box element in the promoter of aromatase gene (38). Finally intrauterine position effects could result in differing

hormonal environment for developing fetuses depending on the sex of neighboring fetuses. Studies demonstrate that the volume of sexual dimorphic nucleus-preoptic area in male rats which are gestated between two male fetuses are 2-fold larger than those gestated between two female fetuses (39).

Similarly, the global *Bmal1*$^{-/-}$ female mice could be divided into two groups: the estrus-cycling group and non-cycling group. The non-cycling females tend to display smaller ovaries than the cycling females. I speculate that the non-cycling global *Bmal1*$^{-/-}$ females could result from insufficient prenatal exposure to estrogen. There is report that female fetuses developing between two males tend to show masculinized anatomical, physiological and behavioral traits as adults (40).

I propose to treat *Bmal1*$^{+/-}$ dams with testosterone or estrogen. The global *Bmal1*$^{-/-}$ daughters of estrogen treated *Bmal1*$^{+/-}$ dams are expected to be all cycling. The global *Bmal1*$^{-/-}$ sons of testosterone treated *Bmal1*$^{+/-}$ dams are expected to have rescued mounting behaviors and thus can sire litters.

BMAL1 AND INFLAMMATION

The double mutant *Bmal1*$^{fx/fx}$*Cre*^{alb}*Lipc*$^{-/-}$ mice are hypothesized to be protected from inflammation in liver. Deletion of *Bmal1* in hepatocyte resulted in higher inflammation and lipoprotein lipase (*lpl*) and hepatic lipase (*lipc*) gene expression (appendix II). Interestingly hepatic lipase-defeciency can protect mouse liver from inflammation (41). In order to test the hypothesis that Bmal1 regulation inflammation process through downregulating hepatic lipase in the liver (41, 42), I propose to generate double mutant mice with hepetic lipase knockout (43)

and hepatocyte-specific *Bmal1* deficiency. The double mutant mice are expected to be protected from hepatic inflammation.

DISRUPTED CIRCADIAN RHYTHMS AND TUMORIGENESIS

The tumor-growth in *Bmal1*$^{fx/fx}$*Cre*^{Syt10} mice is hypothesized to be faster than Syt10/cre mice. Tumor-growth was shown to be faster in mice with lesioned SCN than in controls for two tumor models, transplanted Glasgow osteosarcoma and pancreatic adenocarcinoma (44). Syt10/Cre directs Cre deletion in SCN (18). We propose to transplant Syt10/cre or *Bmal1*$^{fx/fx}$*Cre*^{Syt10} mice Glasgow osteosarcoma or pancreatic adenocarcinoma.

REFERENCES

1. Quennell JH*, et al.* (2009) Leptin indirectly regulates gonadotropin-releasing hormone neuronal function. *Endocrinology* 150(6):2805-2812.
2. Burgin KE*, et al.* (1990) In situ hybridization histochemistry of Ca2+/calmodulin-dependent protein kinase in developing rat brain. *J Neurosci* 10(6):1788-1798.
3. Galichet C, Lovell-Badge R, & Rizzoti K (2010) Nestin-Cre mice are affected by hypopituitarism, which is not due to significant activity of the transgene in the pituitary gland. *PLoS One* 5(7):e11443.
4. Chappell PE, White RS, & Mellon PL (2003) Circadian gene expression regulates pulsatile gonadotropin-releasing hormone (GnRH) secretory patterns in the hypothalamic GnRH-secreting GT1-7 cell line. *J Neurosci* 23(35):11202-11213.
5. Khan AR & Kauffman AS (2012) The role of kisspeptin and RFamide-related peptide-3 neurones in the circadian-timed preovulatory luteinising hormone surge. *J Neuroendocrinol* 24(1):131-143.
6. Plant TM (2012) A comparison of the neuroendocrine mechanisms underlying the initiation of the preovulatory LH surge in the human, Old World monkey and rodent. *Front Neuroendocrinol*.
7. Smarr BL, Morris E, & de la Iglesia HO (2012) The Dorsomedial Suprachiasmatic Nucleus Times Circadian Expression of Kiss1 and the Luteinizing Hormone Surge. *Endocrinology*.
8. Williams WP, 3rd, Jarjisian SG, Mikkelsen JD, & Kriegsfeld LJ (2011) Circadian control of kisspeptin and a gated GnRH response mediate the preovulatory luteinizing hormone surge. *Endocrinology* 152(2):595-606.
9. Gerhold LM, Rosewell KL, & Wise PM (2005) Suppression of vasoactive intestinal polypeptide in the suprachiasmatic nucleus leads to aging-like alterations in cAMP rhythms and activation of gonadotropin-releasing hormone neurons. *J Neurosci* 25(1):62-67.
10. Xu Z*, et al.* (2011) Circadian transcriptional factor DBP regulates expression of Kiss1 in the anteroventral periventricular nucleus. *Mol Cell Endocrinol* 339(1-2):90-97.
11. Kriegsfeld LJ, Silver R, Gore AC, & Crews D (2002) Vasoactive intestinal polypeptide contacts on gonadotropin-releasing hormone neurones increase following puberty in female rats. *J Neuroendocrinol* 14(9):685-690.
12. Klingman KM, Marsh EE, Klerman EB, Anderson EJ, & Hall JE (2011) Absence of circadian rhythms of gonadotropin secretion in women. *J Clin Endocrinol Metab* 96(5):1456-1461.
13. Mayer C & Boehm U (2011) Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nat Neurosci* 14(6):704-710.
14. Piper ML, Unger EK, Myers MG, Jr., & Xu AW (2008) Specific physiological roles for signal transducer and activator of transcription 3 in leptin receptor-expressing neurons. *Mol Endocrinol* 22(3):751-759.
15. Klebanov S, Astle CM, DeSimone O, Ablamunits V, & Harrison DE (2005) Adipose tissue transplantation protects ob/ob mice from obesity, normalizes insulin sensitivity and restores fertility. *J Endocrinol* 186(1):203-211.
16. Mason MM, He Y, Chen H, Quon MJ, & Reitman M (1998) Regulation of leptin promoter function by Sp1, C/EBP, and a novel factor. *Endocrinology* 139(3):1013-1022.
17. Mieda M & Sakurai T (2011) Bmal1 in the nervous system is essential for normal adaptation of circadian locomotor activity and food intake to periodic feeding. *J Neurosci* 31(43):15391-15396.
18. Husse J, Zhou X, Shostak A, Oster H, & Eichele G (Synaptotagmin10-Cre, a driver to disrupt clock genes in the SCN. *J Biol Rhythms* 26(5):379-389.
19. Duan J*, et al.* (2007) Effects of subcutaneous leptin injections on hypothalamic gene profiles in lean and ob/ob mice. *Obesity (Silver Spring)* 15(11):2624-2633.

20. Fick LJ, Fick GH, & Belsham DD (2011) Palmitate alters the rhythmic expression of molecular clock genes and orexigenic neuropeptide Y mRNA levels within immortalized, hypothalamic neurons. *Biochem Biophys Res Commun* 413(3):414-419.
21. Sainsbury A*, et al.* (2002) Y4 receptor knockout rescues fertility in ob/ob mice. *Genes Dev* 16(9):1077-1088.
22. Gamble KL, Paul KN, Karom MC, Tosini G, & Albers HE (2006) Paradoxical effects of NPY in the suprachiasmatic nucleus. *Eur J Neurosci* 23(9):2488-2494.
23. Fick LJ, Fick GH, & Belsham DD (2010) Rhythmic clock and neuropeptide gene expression in hypothalamic mHypoE-44 neurons. *Mol Cell Endocrinol* 323(2):298-306.
24. Besing RC*, et al.* (2012) Neuropeptide Y-induced phase shifts of PER2::LUC rhythms are mediated by long-term suppression of neuronal excitability in a phase-specific manner. *Chronobiol Int* 29(2):91-102.
25. Chao PT, Yang L, Aja S, Moran TH, & Bi S (2011) Knockdown of NPY expression in the dorsomedial hypothalamus promotes development of brown adipocytes and prevents diet-induced obesity. *Cell Metab* 13(5):573-583.
26. Glass JD, Guinn J, Kaur G, & Francl JM (2010) On the intrinsic regulation of neuropeptide Y release in the mammalian suprachiasmatic nucleus circadian clock. *Eur J Neurosci* 31(6):1117-1126.
27. Dyzma M, Boudjeltia KZ, Faraut B, & Kerkhofs M (2010) Neuropeptide Y and sleep. *Sleep Med Rev* 14(3):161-165.
28. Sainsbury A*, et al.* (2002) Y4 receptor knockout rescues fertility in ob/ob mice. *Genes Dev* 16(9):1077-1088.
29. Son GH*, et al.* (2008) Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proc Natl Acad Sci U S A* 105(52):20970-20975.
30. Alvarez JD*, et al.* (2008) The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J Biol Rhythms* 23(1):26-36.
31. Bingham NC, Verma-Kurvari S, Parada LF, & Parker KL (2006) Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44(9):419-424.
32. Schroeder A, Loh DH, Jordan MC, Roos KP, & Colwell CS (2011) Circadian regulation of cardiovascular function: a role for vasoactive intestinal peptide. *Am J Physiol Heart Circ Physiol* 300(1):H241-250.
33. Lacombe A*, et al.* (2007) Lack of vasoactive intestinal peptide reduces testosterone levels and reproductive aging in mouse testis. *J Endocrinol* 194(1):153-160.
34. Boer K, Boer GJ, & Swaab DF (1981) Reproduction in Brattleboro rats with diabetes insipidus. *J Reprod Fertil* 61(2):273-280.
35. Manson JE (2008) Prenatal exposure to sex steroid hormones and behavioral/cognitive outcomes. *Metabolism* 57 Suppl 2:S16-21.
36. Brockman R, Bunick D, & Mahoney MM (2011) Estradiol deficiency during development modulates the expression of circadian and daily rhythms in male and female aromatase knockout mice. *Horm Behav* 60(4):439-447.
37. Fisher CR, Graves KH, Parlow AF, & Simpson ER (1998) Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc Natl Acad Sci U S A* 95(12):6965-6970.
38. Shimizu T*, et al.* (2011) Circadian Clock genes Per2 and clock regulate steroid production, cell proliferation, and luteinizing hormone receptor transcription in ovarian granulosa cells. *Biochem Biophys Res Commun* 412(1):132-135.

39. Pei M, Matsuda K, Sakamoto H, & Kawata M (2006) Intrauterine proximity to male fetuses affects the morphology of the sexually dimorphic nucleus of the preoptic area in the adult rat brain. *Eur J Neurosci* 23(5):1234-1240.
40. Ryan BC & Vandenbergh JG (2002) Intrauterine position effects. *Neurosci Biobehav Rev* 26(6):665-678.
41. Chiu HK*, et al.* (2010) Mice lacking hepatic lipase are lean and protected against diet-induced obesity and hepatic steatosis. *Endocrinology* 151(3):993-1001.
42. Mead JR & Ramji DP (2002) The pivotal role of lipoprotein lipase in atherosclerosis. *Cardiovasc Res* 55(2):261-269.
43. Homanics GE*, et al.* (1995) Mild dyslipidemia in mice following targeted inactivation of the hepatic lipase gene. *J Biol Chem* 270(7):2974-2980.
44. Filipski E*, et al.* (2003) Disruption of circadian coordination accelerates malignant growth in mice. *Pathol Biol (Paris)* 51(4):216-219.

Appendix I

BACKGROUND

There is considerable evidence for a strong relationship between circadian rhythms and cancer. First, Epidemiological evidence has suggested a relationship between circadian rhythms and human cancer incidence. That is, individuals with activity rhythms that diverge from human norms, such as shift workers, cabin crew, and nurses, are at a higher risk for breast, colon, and prostate cancer (1-4). In addition, obese women working rotating night shifts doubled their baseline risk of endometrial cancer (multivariate relative risks, 2.09; 95% CI, 1.24-3.52) compared with obese women who did no night work. Second, altered circadian gene (*PER2*) expression has been found in breast cancer. Altered PER2 levels in breast cancers have been associated with altered methylation of the *PER2* promoter (5). Finally, mutation in the familial *adenomatous polyposis coli* (*APC*) gene and deregulated Wnt-APC-β-catenin signaling pathway contributes to the development of spontaneous and hereditary colorectal cancer. *Per2* mutation induces tumorigenesis in the murine model of familial adenomatous polyposis, the $Apc^{Min/+}$ (6).

METHODS AND RESULTS

Our lab has developed the genetic tools by which we can manipulate the circadian clock in the murine system. Through targeted mutations at the *Bmal1* locus and use of tissue-specific Cre-mediated deletion, we can generate strains of mice where the clock can be disrupted globally or in specific cell types.

Bmal1$^{+/-}$*Apc*$^{Min/+}$ males were mated to *Bmal1*$^{+/-}$ females. Offspring were killed at three months of age and colon tumors were counted. We observed that *Bmal1*$^{-/-}$*Apc*^{min} mice developed significantly more colon tumors than their littermate *Bmal1*$^{+/\pm}$*Apc*^{min} controls (Figure A-1), suggesting that the global deletion of *Bmal1* does accelerate colon tumorigenesis.

To investigate if conditional deletion of *Bmal1* in hepatocytes would accelerate liver tumorigenesis, we used the lox P- Cre system and a mouse line expressing Cre under the control of the Albumin promoter. *Bmal1*$^{fx/fx}$ or *Bmal1*$^{fx/fx}$*Cre*^{alb} male mice were initiated with 0.1 umole DEN/gram body wt given IP in tricaprylin (10 ul/gram body wt) at 13d of age. At 9-10 weeks of age, biweekly treatment with TCDD, (10 ug/kg in corn oil by gavage) began. Controls received corn oil, 10 ml/kg. Livers were harvested and scored at 24 weeks. No difference was seen in tumor multiplicity between *Bmal1*$^{fx/fx}$ or *Bmal1*$^{fx/fx}$*Cre*^{alb} male mice.

References

1. Lie JA*, et al.* (2011) Night work and breast cancer risk among Norwegian nurses: assessment by different exposure metrics. *Am J Epidemiol* 173(11):1272-1279.
2. Kubo T*, et al.* (2006) Prospective cohort study of the risk of prostate cancer among rotating-shift workers: findings from the Japan collaborative cohort study. *Am J Epidemiol* 164(6):549-555.
3. Schernhammer ES*, et al.* (2003) Night-shift work and risk of colorectal cancer in the nurses' health study. *J Natl Cancer Inst* 95(11):825-828.
4. Lie JA, Roessink J, & Kjaerheim K (2006) Breast cancer and night work among Norwegian nurses. *Cancer Causes Control* 17(1):39-44.
5. Chen ST*, et al.* (2005) Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers. *Carcinogenesis* 26(7):1241-1246.
6. Wood PA*, et al.* (2008) Period 2 mutation accelerates ApcMin/+ tumorigenesis. *Mol Cancer Res* 6(11):1786-1793.

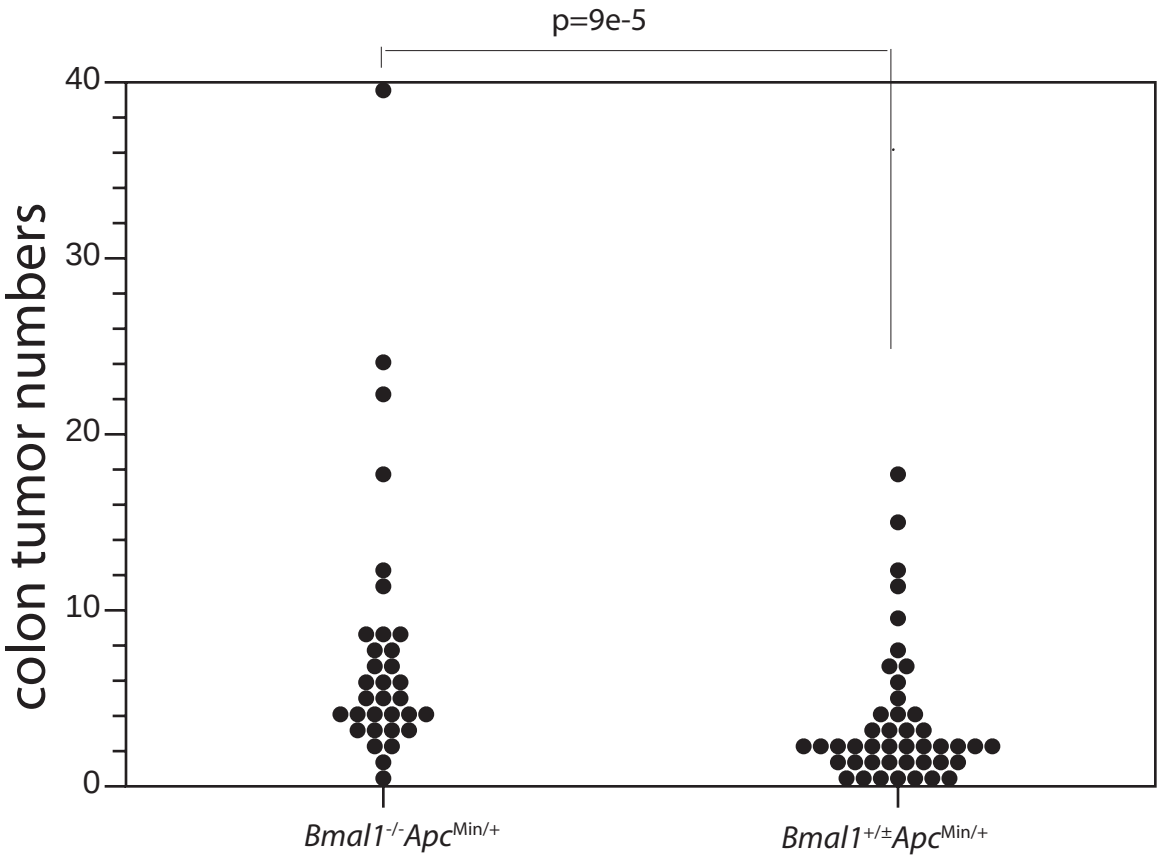


Figure A-1

Appendix II

CONDITIONAL BMAL1 DELETION IN HEPATOCYTES LEADS TO INFLAMMATION ON AGING LIVER

Chronic disruption of circadian rhythm leads to higher risk of inflammation(1). We generated conditional Bmal1 knockout mice by using loxP-Cre system and the Cre mouse line diven by Albumin promoter. Aged (600 days) *Bmal1*$^{fx/fx}$*Cre*^{Alb} mice displayed evidence of increased inflammation by H+E staining (Figure A-2a) or F8/40 staining (Figure A-2b).

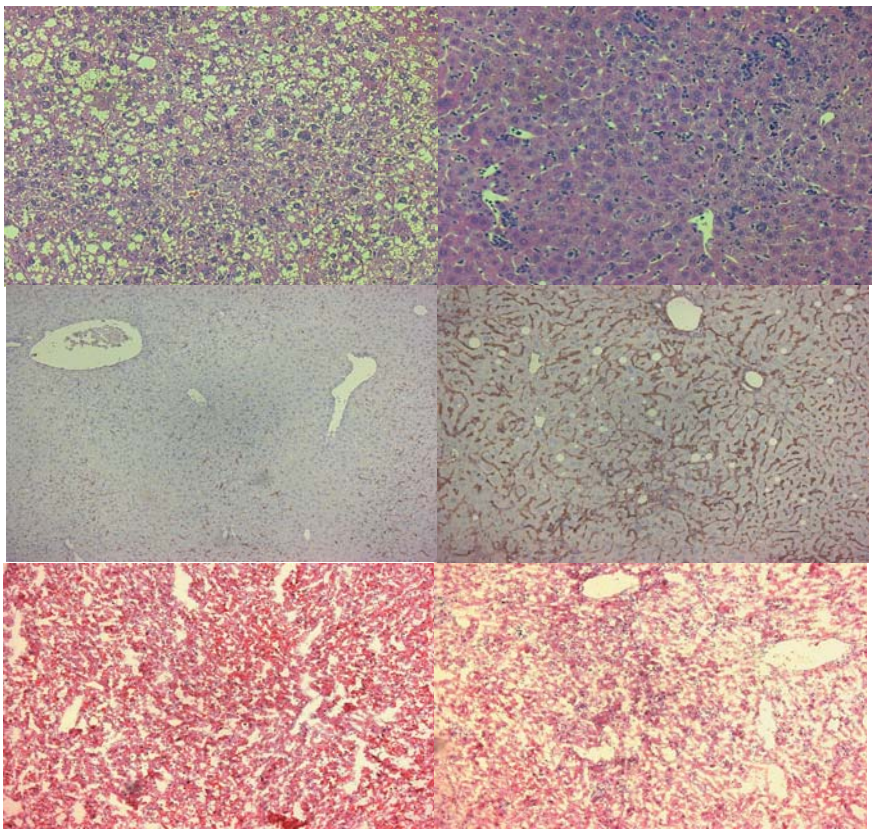
We profiled gene expression in the livers of *Bmal1*$^{fx/fx}$*Cre*^{Alb} males by microarray analysis. Three *Bmal1*$^{fx/fx}$*Cre*^{Alb} or *Bmal1*$^{fx/fx}$ mice were killed every 4 hours from zt 0 to zt 20. Livers were collected and RNA was prepared. Three RNA samples from each time point for each genotype were hybridized against a common reference RNA sample on each Agilent mouse whole genome microarray (GF4122F, Agilent technologies). This profiling was repeated for another independent day and on the *Bmal1*$^{-/-}$ and *Bmal1*$^{+/+}$ male mice.

We found that in both *Bmal1*$^{fx/fx}$*Cre*^{Alb} and *Bmal1*$^{-/-}$ male mice lipolipase (lpl) and hepatic lipase (lipc) expression is upregulated compared to *Bmal1*$^{fx/fx}$ *and Bmal1*$^{+/+}$ mice respectively (Figure A-2d). We observed that there is more lipid accumulation in *Bmal1*$^{fx/fx}$ than *Bmal1*$^{fx/fx}$*Cre*^{Alb} livers by oil red O staining (Figure A-2c).

Hepatic lipase deficiency reduced adipose tissue macrophage content (2). We hypothesize that upregulated lipase gene expression might induce inflammation in *Bmal1*$^{fx/fx}$Cre^{alb} mice.

REFERENCES

1. Castanon-Cervantes O*, et al.* (2010) Dysregulation of inflammatory responses by chronic circadian disruption. *J Immunol* 185(10):5796-5805.
2. Chiu HK*, et al.* (2010) Mice lacking hepatic lipase are lean and protected against diet-induced obesity and hepatic steatosis. *Endocrinology* 151(3):993-1001.



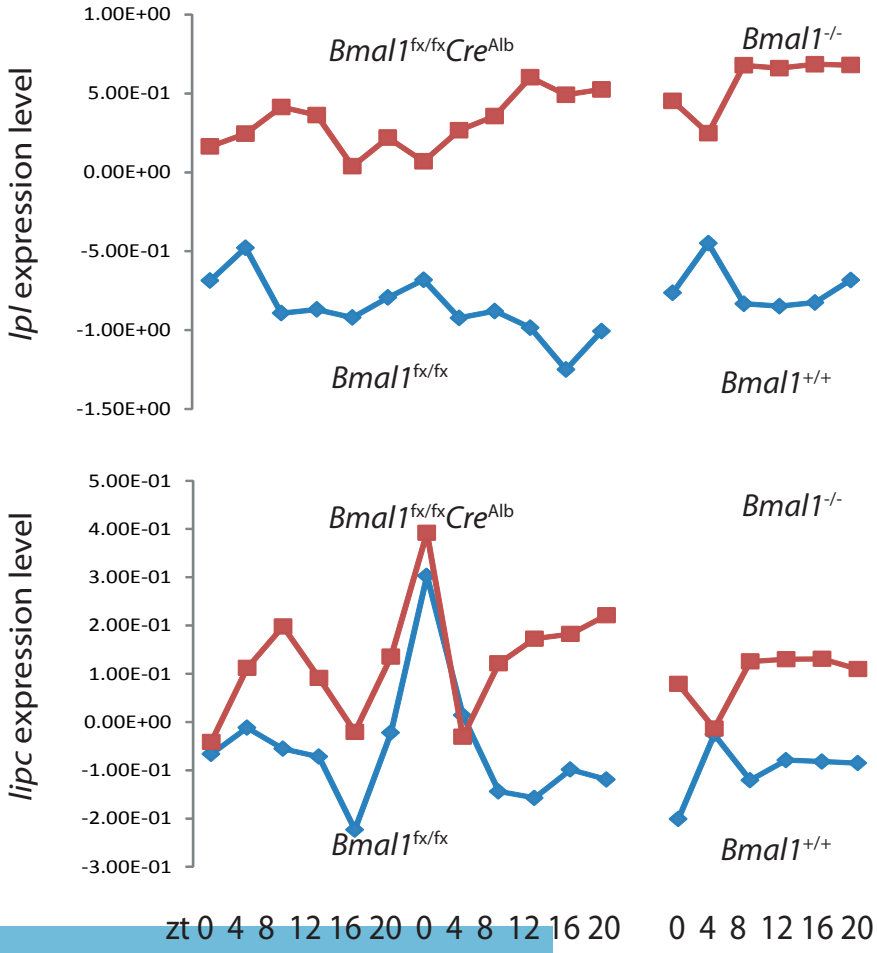


Figure A-2