THE UNIVERSITY OF CHICAGO

CIRCADIAN INFLUENCES ON THE FUNCTIONAL REGULATION OF THE REPRODUCTIVE AND IMMUNE SYSTEMS OF SIBERIAN HAMSTERS (*PHODOPUS SUNGORUS*)

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ERIN JENNA CABLE

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Abstract

Circadian timing of behavior and physiology has direct implications for both survival and reproduction. A circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN) coordinates the daily rhythms observed in most all aspects of biology and behavior, including reproductive and immune function. However, the precise mechanisms by which the SCN influences and interacts with these systems is not entirely clear. In these experiments we tested several hypotheses relevant to unresolved questions in the complex and bidirectional interactions between the circadian, reproductive and immune systems. In Chapter 3 we utilized a novel model of circadian arrhythmia in Siberian hamsters (*Phodopus sungorus*) to examine the consequences of circadian disruption on reproductive neuroendocrine function and fertility. In Chapter 4 we employed this model of circadian arrhythmia to determine whether circadian disruption compromised a major aspect of a coordinated immune response (cutaneous wound healing). Finally, in Chapter 5 we examined the molecular mechanisms by which the innate immune response to infection mediates suppression of reproductive function. Together, the work confirms and extends an emerging theme that the circadian system plays a pivotal role in the coordination and mediation of normal immune and reproductive function, and identifies novel phenomenology and mechanisms by which the circadian system may accomplish these effects.

Chapter 1: General Introduction and Background

Most behavior, whether solitary or social, must be coordinated in time. Absent the capacity to accurately predict information about time of day and time of year, and coordinate physiology and brain function accordingly, behaviors and physiological events essential to survival (reproduction, exploratory behavior, immune function) risk being engaged at times of year that are suboptimal (at best), and detrimental to survival (at worst). From daily cycles of temperature and predation, to seasonal cycles of humidity and food availability, the timing of behavior has consequences for fitness. Absent the ability to orient in time, interactions between individuals and their environment would be impossible to coordinate. With few exceptions, organisms use light information in the environment to coordinate external behaviors, and internal physiology. Abundant work over the past 40 years has identified multiple mechanisms (neural, humoral) by which the central nervous system (CNS) perceives and processes time information from the external world. Equally important is the issue of how daily and annual time information, once encoded in the CNS, is then communicated to the periphery, in order to adjust behavior and physiology in an adaptive fashion.

In an optimal environment, an organism can maintain a constant and stable relation to the circadian environment. Conditions are not always optimal, however. The mechanisms for circadian adaptation to changes in the external environment have been defined over the course of the last 50 years, but what remains to be fully defined are the systemic consequences of acute and chronic circadian disruption on the bidirectional interactions between the central circadian pacemaker, and downstream central and peripheral systems. The focus of these experiments specifically examines how the reproductive and immune systems compensate for and recover

from repeated circadian disruption. In order to fully appreciate these questions, it is essential to consider the dynamic relationships between these three systems under undisturbed conditions.

1.1 Homeostasis and biological rhythms

Homeostasis is the maintenance of a static, optimal, set-point in physiological state. Well-known examples of homeostatic mechanisms include temperature regulation, fluid concentration and metabolism- although this is by no means a complete list. Because the external environment is variable, these homeostatic mechanisms must have the capacity to reliably interpret external conditions and adapt behavioral and physiological response to defend the optimal condition. A common example, internal body temperature in mammals, must be maintained in a relatively small window for survival (Kurz, 2008). Seasonal and daily changes in external environmental temperature require the activation of homeostatic mechanisms to maintain internal core temperature. Acting as a negative feedback loop, these mechanisms will continue until the desired set-point has been reached, at which point they will cease. To maintain body temperature the organism engages in behaviors conducive to desired state: seeking shade to cool, or huddling to warm. Internally, physiological mechanisms will activate, such as perspiration for cooling, and shivering for warming (Kurz, 2008). Together, these mechanisms are remarkably successful in defending optimal conditions of internal state.

The concept of homeostasis seems to be initially incompatible with biological rhythms, which introduces a fundamental variability of internal physiology. Biological rhythms are oscillations of internal function, completing each cycle of variability with a set period of time. Circadian rhythms have a period of approximately 24 hours, while circannual rhythms oscillate with a period of approximately one year. The mechanisms for each will be discussed in the

following sections. Biological rhythms are present in almost every aspect of behavior and physiology (Sharma, 2003). Homeostatic mechanisms and biological rhythms exist simultaneously within the same organism, the result being that the set-point for most functions is not at all static. The desired homeostatic state changes in a predictable manner, based on the current phase of the biological rhythm. It is this predictable nature of biological rhythms that permits a cohesive relationship between homeostasis and circadian variation. Using temperature as an example again, core internal temperature oscillates in most mammalian species with a circadian period. If an external disturbance requires compensatory intervention from homeostatic mechanisms, these mechanisms will engage to defend the temperature set-point desired *not* at the time of disruption, but the anticipated set-point expected based on the continuing rhythm in body temperature (Moore-ede, 1986). The interaction between these two systems to achieve optimal internal conditions is a well-defined example of allostasis, the maintenance of stability through change (McEwen and Karatsoreos, 2015).

Biological rhythms are an adaptation to constant, but predictable, environmental variation. Daily and seasonal changes in external conditions operate with reliable rhythmicity, but must be anticipated by the organism through changes in internal physiology. Disruption to the predictable variation in daily rhythms has robust negative consequences on an organism, as internal mechanisms struggle to adjust to the unexpected environmental conditions. A popular example of this is sudden change in light-dark rhythms due to long distance travel, or jet-lag. While acute exposure to jet-lag is not irreconcilable to the circadian system, repeated exposure severely increases morbidity in aged mice, absent additional stressors (Davidson et al., 2006). Mice in this study were maintained in a single home cage, and were exposed only to changes in lighting conditions mimicking a 6 hour east or west jet-lag once per week for 8 weeks. By the

conclusion of the study, only 47% of phase advanced (east-ward travel) and 68% of phase delayed (west-ward travel) mice survived, compared to 83% of un-shifted mice (Davidson et al., 2006). Such an outcome is dramatic, but not unique. Disruption of circadian function has been frequently shown to negatively interfere with normal physiology, as will be discussed below and throughout the following chapters. Acute and chronic circadian disruption increases allostatic load of the organism, generating "wear and tear" on homeostatic mechanisms over the course of the lifespan (McEwen et al., 2006; McEwen and Karatsoreos, 2015). How increased allostatic load due to circadian dysregulation manifests as wear and tear in the reproductive and immune systems is a primary question of this work.

1.2 Circadian Rhythms

The circadian clock is a pacemaker, defined as a substrate that sets the period and phase of subordinate oscillators. The hypothalamic suprachiasmatic nucleus (SCN) receives time information (via direct retino-hypothalamic projections) that allows it to engage resetting responses of the circadian clock, and the SCN also generates output that permits organismal orientation to a 24 hour, or daily timescale. Because in nature, light information is the most invariable, faithful representation of time of day, it is the primary *zeitgeber* (time giver) for the circadian system in most organisms (Aschoff, 1960). Other environmental cues, such as temperature or food availability, also oscillate on ~24 hour scales, but exhibit much more variability as compared to light information. Absent a precise *zeitgeber*, the master circadian pacemaker (defined further below) is still functional, but does not entrain (synchronize its timekeeping process) with the external environment, and instead exhibits its endogenous free-running period (Bruce and Pittendrigh, 1957; Moore-ede et al., 1982). The interval required to

complete a full circadian oscillation is referred to as the circadian 'tau'. Absent an external environmental cue, the endogenous tau typically deviates from a precise 24 hour oscillation. Depending on the species and the individual, endogenous tau is slightly greater or less than 24 hours. Light information entrains the circadian system by resetting the endogenous clock (changing tau) on a daily basis, to a period of 24 hours, (equal to that of the zeitgeber), via either phase advancing (speeding up) or phase delaying (slowing down) the clock-- entrainment of behavior, neural, and endocrine function to the 24 hour day ensues (Pittendrigh and Daan, 1976, Moore-ede et al., 1982). The mechanisms through which light is processed by the circadian system to mediate entrainment is detailed in the sections below. The ability of the CNS to determine environmental time and adjust behavior adaptively is a hallmark example of how brain function is affected by the external environment, and how neural substrates exhibit plastic responses to the environmental context.

1.3 Circadian Inputs

The processing of environmental time information begins in the eye. Light information is received at the retina, and along with normal visual stimulation via rods and cones, intrinsically photosensitive retinal ganglion cells (ipRGCs), which are characterized by expression of the photopigment melanopsin, are specifically sensitive to circadian light information (Berson, Dunn and Takao, 2002; Hattar et al., 2002; Panda et al., 2003): melanopsin-containing ipRGCs are slow adapting, and non-image forming (Morin and Allen, 2005). Along with rods and cones, activation of ipRGCs communicate light intensity to the SCN via the retinohypothalamic tract (RHT), a neural pathway which diverges from the optic tract at the level of the optic chiasm, and makes synapses in the SCN and the intergeniculate leaflet (IGL) of the lateral geniculate nucleus

(LGN; Morin and Allen, 2005). The visual pathway from the retina directly to the SCN is physiologically distinct from the image-forming visual pathway, which communicates light information from rods and cones, via ganglion cells to other specialized geniculate, and later, cortical targets. Thus, even at the first relays of processing, time information derived from light is distinct from other types of visual processing.

A caveat here is that although melanopsin-containing ipRGCs and the RHT are the most direct route in transmitting light to the SCN, it is not the only pathway by which this occurs. Mice lacking ipRGCs are still capable of behavioral entrainment to a 24 h light-dark cycle (Panda et al., 2003). Only when ipRGCs and rods/cones are eliminated do mice fail to entrain behavioral circadian rhythms to light (Panda et al., 2003). Thus, both the melanopsin and rod/cone visual pathways are sufficient to communicate light information to the circadian clock, and neither is independently necessary. This demonstrates a degree of redundancy in circadian light inputs; such redundancy emphasizes the importance of ensuring circadian entrainment as an adaptive mechanism.

1.4 The suprachiasmatic nucleus: a circadian pacemaker

Circadian rhythms (CRs) in behavior, hormone secretion, and neural activity are driven by the SCN, the master circadian pacemaker. The SCN entrains to the daily light-dark cycle (via photic input pathways elaborated in the previous section), and it determines the period and phase of organismal-level biological rhythms. Defining the SCN as the physiological basis for the circadian pacemaker required convergent evidence that established both its necessity and sufficiency. Complete bilateral lesions of the SCN (SCNx) resulted in a loss of locomotor, drinking, and endocrine rhythms (Stephan and Zucker, 1972; Moore and Eichler, 1972). These

data clearly established *necessity* of the SCN in the generation of CRs. Subsequent work demonstrated that the SCN is also *sufficient* to generate CRs. A mutant line of Syrian hamsters (called "tau mutants") generate CRs with an endogenous period of ~20 h, rather than the 24 h rhythms that are more typical of this species (Ralph and Menaker, 1988). When fetal SCN tissue from either wild-type (WT; hamsters with CRs of ~24 h) or tau mutant donor hamsters were transplanted into the third ventricle of arrhythmic SCNx hamsters, the donor tissue restored locomotor rhythms in WT hamsters, but at the period of the donor tissue (Ralph et al., 1990; LeSauter et al., 1996). Interestingly, not all rhythms were restored following the transplantation of SCN tissue (Meyer-Bernstein et al., 1999). Locomotor activity (wheel running) was restored by the transplants, however, many other SCN outputs (all hormonal rhythms, for example) did not recover following transplantation. The lack of complete CR recovery following SCN tissue donation indicates that functional connectivity in the original SCN-output network cannot be fully regenerated in this model, and has yielded the currently-held perspective that the SCN communicates circadian information to extra-SCN neurological systems via both diffusible (humoral) signals, and via neural connections. Donated SCN tissue rescues circadian behavior in systems dependent on diffusible factors, but transplanted SCN tissue makes few, if any, functional synapses (Lehman et al., 1987) and is unable to restore the original connectivity necessary for those systems (LeSauter et al., 1996; Silver et al., 1996; Guo et al., 2005).

The mechanism by which light information is processed by the mammalian SCN to maintain time information in the CNS involves changes in the expression of a collection of genes and their protein products which comprise a positive and negative feedback loop, creating a molecular clock within each cell (a transcriptional-translational feedback loop; TTFL; Maywood et al., 2007). The interval required to complete one cycle of this molecular loop is approximately

24 hours in WT organisms; this generates the ~24 h endogenous tau. Exact 24 hour cycles in light stimulation (*zeitgebers*) entrain/synchronize the molecular clock to a 24 hour day. Briefly, within the nucleus of the cell, two genes, *Circadian Locomotor Output Cycles Kaput (Clock*) and *Brain Muscle ARNTL Like 1 (Bmal1*), proteins (CLOCK and BMAL1) heterodimerize, and bind to E-boxes on the promotor regions of the *Period (Per*) and *Cryptochrome (Cry*) genes, stimulating their transcription. Once transcribed, PER and CRY proteins exit the nucleus and herterodimerize in the cytoplasm. After PER/CRY are phosphorylated, the complex re-enters the nucleus, and also binds to the E-box on *Per* and *Cry* promotor regions, inhibiting their own transcription. Along with increasing *Per* and *Cry* transcription, the CLOCK/BMAL1 binding to the E-box increases the transcription of *Rev-Erb-α*, which then suppresses the transcription of *Bmal1* (For Review: Reppert and Weaver, 2001; Ko and Takahashi, 2006). Light information reaching the SCN entrains the molecular clock via stimulation of glutamate release from the RHT. The increase in glutamate promotes the transcription of *Per*, thereby shifting the phase of the TTFL, in a direction that facilitates synchrony with external light information (Shigeyoshi et al., 1997). Every nucleated cell in the body, and in the SCN in particular, expresses this TTFL, and it is the coordination between cells within the SCN that create a coherent circadian output, and provide a CNS representation of biological (circadian) time.

How the SCN communicates circadian time information to neurological systems responsible for countless biological processes continues to be a complex question in the field of chronobiology. Indeed, the question is made more complex by the variable ways in which these systems are influenced by the circadian pacemaker. Research to be addressed in this dissertation will consider the circadian influence on several targets critical to survival and adaptation, specifically, the reproductive system and the immune system.

1.5 Seasonal Rhythms

Whereas the circadian clock has been extensively dissected and defined, the CNS tracks time along longer time scales as well. Changes in the environment oscillate on an annual scale as well as a daily scale. Depending on latitude, the amount of daylight seen in a 24 h period can be highly variable depending on the time of year. In addition to changes in day length, temperature, humidity, and food availability also change on an annual basis. The coordination of reproductive efforts (especially in female mammals) such that offspring are issued at energetically-plentiful times of the annual cycle, is critical to survival and adaptation (Bronson, 1985). Seasonal adjustments in the immune system are ubiquitous as well (Nelson and Demas, 1996). Many species survive and adapt to the annual changes in environmental conditions, and do so by engaging annual timing mechanisms, capable of measuring intervals that approximate one year (much in the same way that the circadian clock approximates an interval of one day) in order to coordinate the seasonal changes necessary for survival in seasonally-variable environmental conditions.

The aforementioned environmental changes trigger direct changes in the physiology of many mammals. Siberian hamsters (*Phodopus sungorus*) are one such seasonally diphenic species, and are a canonical model organism for studying the physiology of seasonality. Siberian hamsters are native to Mongolia and Northeastern China. This location experiences dramatic seasonal changes in temperature and day length. In summer, ambient temperatures reach 25 ºC with up to 17 hours of light/day. In winter, temperatures are as low as -25 ºC, and days are short (7 h light/day; Weiner and Heldmaier, 1987). These seasonal changes in environmental conditions have favored the evolution of strikingly adaptive seasonal changes in behavior and

physiology in hamsters. Such changes can be mimicked in the laboratory with the mere manipulation of day length (photoperiod) alone. When transferred from long summer days to short winter days in the laboratory, hamster body weight decreases by approximately 30%, food intake decreases by approximately the same amount, fur pelage changes from agouti (brownish and tan) to white, and the gonads regress in size, gametogenesis and steroid hormone production is reduced (Bartness and Wade, 1986; Bartness et al., 2002; Prendergast et al., 2002); reproductive behavior also ceases (Zucker, 2001). The change from summer to winter phenotype is achieved over a period of 8-12 weeks in the laboratory under static long (>14 h light/day) and short (<12 h light/day) day lengths. In nature, shortening day lengths following the summer solstice signal the beginning of the phenotypic change in anticipation of winter-like conditions (Gorman, 1995; Gorman and Zucker, 1995; Butler et al., 2007; Butler and Zucker, 2009; Paul et al., 2008). The mechanisms underlying processing of seasonal environmental signals used to initiate this series of physiological and behavioral changes are essential to an understanding of how the circadian and seasonal system interact.

Different species generate circannual rhythms via markedly different mechanisms. One class of circa-annual timing mechanisms, for example, involves the output of an endogenous, self-sustaining circannual pacemaker. Even in the lab, absent seasonal changes in environment, species such as the golden-mantled ground squirrel exhibit spectacular annual rhythms in hibernation (Pengelley et al., 1976) and body mass (Ruby et al., 1998), indicating the presence of an endogenous circannual clock. A discussion of the physiology of such 'true' circannual timing systems is beyond the scope of this dissertation.

A categorically different class of endogenous annual timing mechanisms does not depend on a self-sustained annual pacemaker, but rather relies on the measurement of day length for the

proper phasing of seasonal physiology. The persistence of such rhythms is dependent on the pineal gland. The pineal is an endocrine organ located on the dorsal surface of the brain, and produces melatonin. Melatonin is secreted during the night, and is suppressed by light input (Kasahara et al., 2000). Secretion of melatonin is an output of the circadian clock, and encodes the duration of night under normal conditions, but still secreted with a period of approximately 24h under constant conditions (Drijfhout et al., 1999). It is the *duration* of the melatonin signal, and not the amplitude or concentration, which indicates the length of the dark phase (Goldman and Darrow, 1983; Darrow and Goldman, 1984; Paul et al., 2008). A longer duration melatonin signal indicates a winter, short-day (long-night) photoperiod, while a shorter duration indicates a summer, long-day (short-night) photoperiod. Changes in the duration of the melatonin signal are sufficient to induce a change in the seasonal phenotype of Siberian hamsters. If administered an injection of melatonin 3 h prior to the onset of the dark phase (thereby increasing the duration of the signal by 3 h), hamsters housed in a long day photoperiod will adopt the winter phenotype, even though the actual light input remains unchanged (Goldman et al., 1984). Seasonally responsive animals that have had the pineal gland removed (PINx) lose the capacity to adapt behavior and physiology to changes in day length, and maintain a long-day phenotype even when housed in short-day photoperiods (Hoffman, 1979; Reiter, 1980). However, the winter-like seasonal response can be elicited in PINx animals given a continuous, long duration (>~8 h/night) infusion of melatonin over several weeks (Goldman et al., 1984; Goldman, 1991). Thus, the pineal melatonin signal, which is controlled by the circadian clock is both necessary and sufficient to induce seasonal changes seen in multiple species.

The circadian aspect of the pineal melatonin signal, a nightly rise in melatonin congruent with the onset of darkness, requires an intact and functional SCN. Indeed, melatonin is a highly

reliable marker of the phase of the SCN (Borjigin et al., 2012; Elliott and Tamarkin, 1994). Following a lesion of the SCN, response to changes in day length are disrupted or eliminated in Siberian hamsters, specifically in the reproductive system (Bittman et al., 1979; Bittman et al., 1991). Timed infusions of melatonin are not sufficient to restore the seasonal photoperiod response in SCN lesioned Siberian hamsters (Bartness et al., 1991), indicating that the SCN is both necessary for sending time information to the SCN and necessary for responding to melatonin-based seasonal time information in this species. The biological basis for this form of circannual rhythm is thus not independent of a functional master circadian pacemaker.

1.6 Reproduction

The Siberian hamster has a four-day estrous cycle (Wynne-Edwards and Lisk, 1984), which is largely under the control of both the seasonal and circadian clock. In many rodent species, ovulation and onset of behavioral sexual receptivity are coordinated to occur just before the onset of the active phase (Fitzgerald and Zucker, 1976). On the day of proestrus, ovarian estradiol and progesterone peak; these hormones act in the hypothalamus to sensitize the release of gonadotropin releasing hormone (GnRH) into the median eminence. GnRH then induces a proestrus surge of luteinizing hormone (LH) from the anterior pituitary, stimulating follicle eruption at the ovary (McCarthy & Becker, 2002). The proestrus LH surge is essential for ovulation, and is dependent on a functioning circadian clock in the SCN (Stetson and Watson-Whitmyre, 1976; Lucas et al., 1999). A time-of-day signal from the SCN has been suggested to drive a “circadian gate”, which dictates the temporal window during which the LH surge can occur, and also determines timing of sexual receptivity in female rodents. Everett and Sawyer (1950) first documented this circadian gate in a classic experiment which demonstrated that a

timed injection of barbiturate on the afternoon of proestrus is sufficient to prevent ovulation in female rats on the ensuing evening, but only does so for 24 hours; the LH surge is merely delayed, but by precisely 24 h. This was the first clear evidence that the circadian clock played a central role in the timing of ovulation on the day of proestrus, as opposed to a four-day clock, entirely separate from the SCN. Fitzgerald and Zucker (1976) added convergent evidence that the master pacemaker in the SCN is the neuroanatomical basis of this 'gate'—this experiment demonstrated that locomotor activity and estrus onset are phase locked in hamsters under constant conditions, suggesting a common pacemaker times both behavioral receptivity and locomotor activity. As mentioned previously, implantation of donor SCN tissue is not sufficient to restore normal rhythms in reproductive hormones (Silver et al., 1996), suggesting that communication between the substrates controlling reproduction in the hypothalamus receive information from the SCN via a neural connection as opposed to a diffusible, humoral signal.

In addition to the proposed circadian gate, the second requirement for a functional surge in LH on the day of proestrus is an increase in ovarian estradiol. At all other times of the estrous cycle, ovarian estradiol exerts negative feedback on GnRH and LH (Blake, 1977). However, during proestrus, the effect of estradiol at the hypothalamic level inverts, and a surge of ovarian estradiol during the appropriate circadian time is necessary to induce the LH surge. This brief and transient change to positive feedback is not well understood, but necessary for the LH surge, and ovulation. Thus, the mechanism by which reproductive function is coordinated requires the integration of both circadian information, and peripheral estradiol feedback.

Only recently have we begun to understand the precise mechanism by which circadian input and ovarian estradiol feedback are coordinated. Initial research focused on GnRH cells within the hypothalamus, as these neurons project directly into the median eminence, and thus to

the anterior pituitary. GnRH neurons do receive input from the SCN via monosynaptic projections from neurons containing vasoactive intestinal polypeptide (VIP), supporting the possibility of GnRH as a locus of circadian input (Van der Beek et al., 1994). However, these cells lack the estrogen receptor alpha (ERα), which mediates the positive feedback effects of ovarian estradiol (Herbison and Theodosis, 1992). Without the capacity to integrate information from peripheral estradiol, it is unlikely GnRH neurons are the unique site of coordination in reproductive function.

Emerging evidence suggests that a population of cells within the dorsomedial hypothalamus (DMH) provides negative feedback on GnRH. Gonadotropin-inhibitory hormone (GnIH) was first discovered in the Japanese quail hypothalamus (Tsutsui et al., 2000), and named due to its capacity to inhibit function in GnRH neurons in a dose dependent manner. A homologue, RFamide-related peptide 3 (RFRP-3), was soon discovered in mammals, and implicated in the inhibition of neural reproductive function in several species. RFRP-3 cells project directly to the pre-optic area (POA), onto GnRH cells (Kriegsfeld et al., 2006). Injections of RFRP-3 are able to rapidly suppress the LH surge (Kriegsfeld et al., 2010). RFRP-3 containing neurons also have ERα receptors, and project to GnRH; thus, they have also been implicated as part of the neural mechanism for *negative* feedback effects of estradiol (Kriegsfeld et al., 2010). In support of this hypothesis, Gibson et al. (2008) found that projections to RFRP-3 neurons from the SCN were inactive during the time of the LH surge, relative to other times in the 24-hour cycle. These data support the proposal that the input from the SCN increases the RFRP-3 inhibitory output. Without active input from the SCN, RFRP-3 may fail to inhibit GnRH, permitting the increase necessary for the LH surge, and ovulation. Although the details of RFRP-3 inhibition and its sensitivity to circadian time are not fully understood, recent evidence

suggests that VIP derived signal from the SCN may mediate RFRP-3 activity in the DMH (Russo et al., 2015). The results of these studies permit the inference that the RF-amides play a central role in the dynamic regulation of the reproductive system over time in females.

1.7 Circadian Disruption and Reproduction

Much of the information about the influence and importance of the circadian system in the optimal reproductive function has come from studies considering the consequences of circadian disruption. There are several methodologies by which the circadian system may become either desynchronized or permanently arrhythmic. Lesions of the SCN, mutations of various clock genes, and long-term exposure to constant bright light all result in significant disruption to the circadian system. While some are temporary and some are permanent, all result in significant disruption to outputs of the central pacemaker, including the coordination of reproductive processes: SCN lesions eliminate ovulation in female rats (Stetson and Watson-Whitmyre, 1976), and induce persistent estrus in golden hamsters (*Mesocricetus auratus*; Brown-Grant and Raisman, 1977; Raisman and Brown-Grant, 1977). Albino rats made arrhythmic via exposure to bright constant light (LL) became anovulatory within 60 days (Brown-Grant, Davidson, and Greig, 1973). Ovulation in LL-treated rats could be induced via coital stimulation; however pregnancy rates were low and rarely yielded viable offspring (Brown-Grant, 1977). In another experiment, degradation of estrous coordination was apparent within 5 cycles following onset of constant bright light, until rats were in a state of persistent estrus (Takeo, 1984).

Transgenic approaches have also provided convergent evidence in support of a key role for the circadian system in female reproduction. Mice bearing mutations in circadian clock genes

exhibit marked decrements in reproductive function, hormone secretion and fertility. Mutations of the *clock* gene caused irregular or extended estrous cycles, and mice with such mutations did not exhibit a clear LH surge on the afternoon of proestrus (Miller et al., 2004). *Clock* mutants did mate and conceive, but prior to parturition had an abnormally high proportion of embryo reabsorption (Miller et al., 2004). The *tau* mutation in the Syrian hamster results in a free running rhythm in LH surge, i.e., it fails to entrain to the external light-dark cycle (Lucas et al., 1999). Mice with a null mutation in the *bmal1* gene exhibit delayed puberty, irregular estrous cycles, embryo lethality and reduced offspring body weight (Boden et al., 2010). Lesions of the SCN, exposure to constant bright light, and mutations of clock-genes resulted in significant disruptions of the reproductive system, consistent with the conjecture that a central representation of time is required for normal LH surges and fertility in mammals.

1.8 Immune Function

In recent decades, it has become more and more appreciated that the immune system does not function independently of the CNS, but rather it is strongly modulated by both the brain and peripheral feedback to the CNS. In classical terms, the immune system is considered to be composed of substrates separate from those that reside in the CNS; although this view has recently received strong challenges (e.g., Louveau et al., 2015). But abundant evidence points to an interaction between the brain and the immune system, regardless of one's perspective on their anatomical independence. Peripheral responses to immune challenges can be altered via lesions to the hypothalamus, indicating a central contribution immunocompetence (Hefco et al., 2004). Additionally, it is well appreciated that stressors, both physical and psychological, impair the response to an immune challenge in multiple species (Dhabhar et al., 1995; Kinsey et al., 2003;

Ebrecht et al., 2004; Gouin and Kiecolt-Glaser, 2011). A clear example of this comes from Kiecolt-Glaser et al. (1995), in which participants responsible for the care of a relative with Alzheimer's disease reported higher levels of stress, and took significantly longer to heal from a small cutaneous wound inflicted via punch biopsy. Besedovsky and del Rey (1996) reviewed hundreds of studies providing evidence for the reciprocal interaction between the immune and endocrine systems. Cells of the immune system express receptors for endocrine products, and both central and peripheral endocrine bodies express immune receptors. Based on the extensive reciprocal interactions with other systems, it is not surprising that the immune system is also heavily engaged in a reciprocal interaction with the circadian system.

1.9 Biological Clocks and Immune Function

Nearly all aspects of immunocompetence vary over the course of the day. Leukocytes in the blood and lymphatic system circulate passively under normal circumstances, and function in both an innate and adaptive capacity to respond to, and eliminate disease or foreign bodies introduced to the system (For review: Maier and Watkins, 1998; Murphy, 2011). Dhabhar et al. (1994) found that multiple leukocyte subpopulations have daily rhythms in tissue 'trafficking'. Leukocyte concentrations are high in the blood during the inactive phase, and concentrations are low during the active phase. Conversely, tissues such as the skin display high leukocyte concentrations during the active phase, and low in the inactive phase (Dhabhar et al., 1994; Viswanathan and Dhabhar 2005). This rhythm can be seen as adaptive, due to the higher risk of tissue damage during the active phase, relative to a time of sedentary behavior. Moreover, during the active phase, individuals are more likely to encounter antigens, thus, more antigen capturing

cells reside in the skin during this phase of the daily cycle and subsequently drain to lymph nodes (for antigen presentation) during the rest phase.

Pathogenic immune challenge directly influences the behavioral outputs of the circadian clock. Administration of lipopolysaccharide (LPS), which simulates a bacterial infection, elicits a suite of behavioral and physiological changes (Maier and Watkins, 1998). LPS is a molecule located on the outer membrane of gram-negative bacteria. When experimentally introduced into an organism, the immune system responds as if to a genuine infection, activating circulating macrophages, which then release proinflammatory cytokines in the periphery (Maier and Watkins, 1998). Increased activation of microglial cells (the resident immune cells in the brain; Hayes, Woodroofe and Cuzner, 1987) results in the increase of proinflammatory cytokines within the CNS (Van Dam et al., 1992; Breder et al., 1994). Specifically involved in this initial inflammatory response is interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα; Dantzer et al., 2000; Konsman et al., 2002). The result of the immunological response is a suite of acute 'sickness behaviors' (Hart, 1990; Kent et al., 1992). These behaviors primarily include fever, reduced locomotor activity, anorexia, anhedonia, social avoidance, and suppression of sex behaviors (Avitsur et al., 1997; Bilbo et al., 2002; Wen and Prendergast, 2007; Yirmiya, 1996).

The severity of the innate immune response is dependent on the circadian phase at which it is introduced. Marpegan et al. (2009) found that LPS injection at the end of the inactive phase generated a larger response of circulating cytokines, and resulted in higher rates of mortality compared to the same dose given in the middle of the active phase. The precise phase of the circadian cycle at which acute phase sickness responses are at their peak depends on many factors, including species, route of administration, and immunological dependent variable

(Scheiermann et al., 2013). As with the reproductive system, the close coordination of the immune response to circadian function suggests a significant vulnerability if such an interaction were to be disturbed (Curtis et al., 2014; review). Gibbs et al. (2012) found markedly exacerbated inflammatory responses in animals and macrophages rendered arrhythmic via mutations of the clock genes *bmal1* and *rev-erb-alpha*. Recent work by Prendergast et al. (2013) has linked circadian disruption to a loss of lymphoid cell trafficking and impaired adaptive immune responses, suggesting that a functional hypothalamic circadian pacemaker is essential for normal organismal-level immunocompetence (see section 1.10).

1.10 Circadian Disruption and Immune Function

As with the reproductive system, a multitude of experiments has demonstrated that disruptions to, or elimination of, normal circadian function results in abnormal immune function. Complete behavioral arrhythmia eliminates normal leukocyte trafficking in Siberian hamsters. Circadian arrhythmic animals have a consistently high level of leukocytes in the blood compared to the daily peak and trough seen in entrained animals (Prendergast et al., 2013). Considering the importance of rapid leukocyte response following immune challenge or tissue damage, a lack of coordinated trafficking may have large negative consequences in the organism (Rojas et al., 2002). In the same model of arrhythmia, animals lacking functional circadian rhythms had a reduced response to delayed-type hypersensitivity (DTH) challenge, compared to an entrained animal (Prendergast et al., 2013). The DTH response is a measure of adaptive immune function. Following initial exposure to a novel antigen, antibodies specific to that compound are created. When challenged with the novel antigen a second time, the subsequent capacity to identify the

antigen and mount an appropriate specific inflammatory response is measured, typically via cutaneous inflammation.

Whereas permanent elimination of circadian function has permitted insight into the organization of the immune system, transient disruptions to the circadian system also result in immune disruption. One of the more dramatic examples of this comes from Castanon-Cervantes et al. (2010), in which mice were placed into a "jet-lag" paradigm prior to immune challenge. Following four 6-hour phase advances, once every week, animals challenged with LPS experienced an 89% mortality rate, compared to 21% in un-shifted mice. The experiment found that sleep quality and cortisol levels were unaffected in the phase-shifted animals, suggesting that the result is due primarily to circadian disruption, and not to secondary effects. Bedrosian et al. (2011) has found that exposing Siberian hamsters to dim light during the dark phase is sufficient to alter both the adaptive and innate immune response following DTH challenge and LPS injection, respectively. Similar to the arrhythmic hamsters, DTH response was diminished in entrained hamsters exposed to dim light at night. Blood samples from these hamsters, taken before and after LPS injection, were plated with bacteria and only able to kill half of the amount of foreign bodies compared to hamsters not exposed to dim light at night (Bedrosian et al., 2011). Together, these data strongly suggest that coordination of the immune system via circadian influence is essential for normal function of the immune response.

1.11 A novel arrhythmia paradigm: The Disruptive Phase Shift Protocol

In order to develop a more complete understanding of both the behavioral and physiological influences of circadian disruption, it is necessary to incorporate the existing data with a methodology of inducing arrhythmia not reliant on traditional manipulations of

physiology (germline mutations, bright LL, brain lesions). Remarkably, in Siberian hamsters, the delivery of a precise combination of nocturnal light treatments permanently eliminates circadian rhythms in sleep/wake, body temperature, and activity (Ruby et al., 1996; Ruby, Barakat, and Heller, 2004). These light treatments also render SCN clock gene expression (*per1*, *per2*, *bmal1*, and *cry1*) arrhythmic via amplitude suppression of the circadian pacemaker (Grone et al., 2011). This noninvasive method has the distinct advantage of allowing animals to remain undisturbed in a standard light-dark cycle, without confounds and limitations of lesions, mutation, or constant bright light. Using this neurologically- and genetically-intact model of circadian arrhythmia, it is possible to investigate the functional consequences of complete circadian desynchrony in both the reproductive and immune system. This approach allow us to gain insights into whether behavioral, reproductive and immune deficiencies commonly observed following classical methods of arrhythmia induction are a cause of the loss of time information per se, or merely a side effect of the method by which the animal was rendered arrhythmic.

1.12 Specific Aims

The present dissertation deploys the DPS model to examine whether reproductive deficits persist in female hamsters following DPS-induced arrhythmia (using several paradigms to test reproductive function; Chapter 3), and whether a major aspect of integrative immune function (wound healing) is compromised following DPS-induced arrhythmia (Chapter 4). Lastly, in light of the clear links among (1) neuroinflammation, (2) hypothalamic regulation of fertility, and (3) the circadian gating of the LH surge, a final experimental chapter (Chapter 5) examines whether neuroinflammation-induced changes in RFamide peptide expression in the hypothalamus mediate the effects of LPS-induced neuroinflammation on reproductive inhibition. Together,

these experiments use arrhythmic (Chapters 3 & 4) and circadian rhythmic (Chapter 5) models of circadian disruption to generate novel insights into the mechanisms by which the circadian system times and influences molecular and behavioral outputs of the reproductive and immune systems. These systems do not act independent of another, but create plastic, bi-directional relationships. Examining the reproductive and immune systems under conditions of a functional and disturbed circadian clock furthers the understanding of how these systems adapt and compensate to increased allostatic load via circadian disruption.

Chapter 2: Methods and Procedures Common to All Experiments

2.1. Animals

All animals in the following experiments were adult (>60 days of age) female, Siberian hamsters (*Phodopus sungorus*) from a breeding colony maintained at the University of Chicago. Hamsters were single-housed in individual polypropylene cages (28 x 17 x 12 cm), on wood shaving bedding (Harlan Sani-Chips, Harlan Inc., Indianapolis, IN, USA) with cotton nesting material. Food (Teklad Rodent Diet 8604, Harlan Inc.) and filtered tap water were provided *ad libitum*. Ambient room temperature was held at a constant 20 ± 0.5°C, with a relative humidity of 53 ± 2%. Hamsters were born into a 15L:9D photoperiod (lights off: 18:00 h CST) until experimental photoperiod treatments were applied. This is a standard long day photoperiod for this species, during which both sexes are reproductively active (Goldman, 2001). All procedures conformed to the USDA guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago.

2.2. Disruptive Phase Shift (DPS)

SCN arrhythmicity was induced in Siberian hamsters using methods developed and optimized by Ruby et al. (2004), and subsequently in our laboratory (Prendergast et al., 2012). Following this paradigm, hamsters were housed for at least three weeks in a 16L:8D photoperiod (lights off: 18:00 h CST). To induce arrhythmia, a two-hour light pulse was administered from the 5th through 7th hours of the 8-h dark phase. On the following day, the 16-h light-dark cycle was delayed by 3 h via extension of the light phase (new lights off: 21:00 h CST). A subset of

control hamsters in the DPS protocol experience only the 3 h phase delay, but not the 2 h light pulse. For all of the following experiments, females were permanently maintained in the new, shifted 16L:8D photoperiod thereafter. At a minimum of three weeks following the DPS procedure, locomotor activity of hamsters was recorded for a minimum of 10 days to identify the behavioral response. Chronotype was categorized as either entrained (ENTR), or arrhythmic (ARR) using criteria described below. Precise age ranges for hamsters during the DPS procedure and subsequent behavioral chronotype analysis will be detailed in the method section for each experiment.

2.3. Locomotor Activity Recording

Passive infrared motion sensors (PIR; Coral Plus, Visonic, Bloomfield, CT) were mounted 22 cm above the floor of the home cage. Motion detectors recorded a movement event when 3 of 27 zones were crossed within the cage. Cumulative locomotor activity was collected into 1-minute bins and recorded using Clocklab software (Actimetrics, Evanston, IL).

2.4. Circadian Chronotyping

Behavioral chronotypes were determined from 10 complete days of PIR activity data. A hamster was considered to be ENTR when onset and offset of locomotor activity occurred consistently at the beginning and end of the dark phase, respectively, following visual inspection of the actogram. χ^2 periodogram analyses were used to confirm entrainment via the presence of a normal significant ($\alpha < 0.001$) peak in the circadian period range. Hamsters were designated as ARR if they exhibited: (1) the absence of clear and significant ($P<0.001$) circadian peaks in χ^2 periodogram, (2) an absence of consistently clear daily activity onsets and offsets, and (3)

locomotor activity distributed throughout the light and dark phases of the LD cycle. Representative actograms of ENTR and ARR hamsters are depicted in Figure 2.1. This noninvasive light-pulse procedure typically renders ≥30% of hamsters behaviorally arrhythmic. Approximately 75% of control hamsters (phase delay, only) re-entrain to the new photocycle (Ruby et al., 2004). Details of animal populations exposed to the DPS procedure varied across experiments, and are reported in the Methods section for each study. Longitudinal analyses of chronotype changes following DPS (absent any other manipulations) have not been explicitly conducted in our lab. The vast majority of individuals that become arrhythmic following DPS remain so indefinitely; the same is true of animals that exhibit the ENTR chronotype. However, a small population of individuals change their chronotype either spontaneously (for unknown reasons) or following experimental manipulations (Wang et al., 2014), thus, chronotype assessments were commonly performed as close in time to experimental manipulations as possible, and, where necessary, locomotor activity monitoring was performed at the end of a study, to confirm maintenance of a specific circadian chronotype throughout an experiment.

Chapter 3: Reproduction and fertility in entrained and arrhythmic Siberian hamsters

3.1 Introduction

The role of the circadian clock in the timing of the 4-day infradian estrous cycle has been acknowledged for the better part of a century. Normal function of the rodent estrous cycle is dependent on a functional circadian system. Disruptions of circadian signaling result in a variety of deficits, the specifics of each being dependent on the manner of circadian disruption (see Section 1.7). The human circadian system has similar influence on reproductive timing (Cahill et al., 1998; Palmer et al., 1982; Renfinetti et al., 2005). It is not surprising that human reproductive function is also vulnerable to disruptions of the clock. Women who experience frequent phase shifts due to transmaridian travel or rotating shift work may have difficulty becoming pregnant or maintaining pregnancy (Bisanti et al., 1996; Infante-Rivard et al., 1993). Maternal circadian disruption is also correlated with low birth weight in infants (Axelsson et al., 1989; Xu et al., 1994). Complete elimination of normal SCN function is not required for the onset of reproductive deficit. Such an outcome indicates a dependence on circadian function, which is compromised under conditions of chronic circadian disruption. Despite the amount of data correlating circadian arrhythmia and reproduction, the neural mechanisms by which the circadian system regulates reproductive function are not fully understood.

Virtually everything currently known about how perturbations of the circadian clock affect female reproductive function has been determined from lesion studies, transgenic/mutant mouse strains, or exposure to bright constant light (LL). While informative and essential to our understanding of circadian integration in reproduction and fertility, each of these techniques is confounded by interpretative limitations. SCN lesions invariably damage surrounding

hypothalamic tissue and fibers of passage (Kriegsfield, LeSauter, and Silver, 2004). Tissues within this area include the DMH, AVPV, and pre-optic area (POA), all of which contain cell populations implicated in the neural regulation of reproductive function (Williams and Kriegsfeld, 2012).

Clock gene mutations are present in all tissues, and are pleiotropic in function- their effects may be due to circadian disruption, but may also result from alterations in, e.g., cellular metabolism (Meyer-Bernstien & Sehgal, 2001). For example, a mutation resulting in the loss of the BMAL1 protein results in severely abnormal circadian function (Bunger et al., 2000), but is also associated with progressive arthropathy of joints over the lifetime. Progressive deterioration of locomotor and reproductive function is not due directly to circadian effects, but to the inability to move normally throughout the environment (Bunger et al., 2005). A mutation of the *clock* gene in mice results in abnormal circadian function most obvious in constant darkness (Vitaterna et al., 2004). Mutation of the *clock* gene also results in decreased metabolic performance. Mice with the *clock* mutation have higher food intake and body mass, resulting in increases in triglycerides, cholesterol, glucose, and leptin concentration (Turek et al., 2005). Such metabolic alterations can negatively affect reproductive function and fertility (Fontana and Torre, 2016; Hassan and Killick, 2004; Schneider, 2004), which would be an indirect effect of circadian clock gene mutation.

Bright LL does not immediately or permanently eliminate entrainment (Depres-Brummer et al., 1995; Eastman et al., 1983) and LL is a stressor, which impairs cognitive function (Ma et al., 2007) and elevates glucocorticoid secretion (Daan and Pittendrigh, 1976). Acute and chronic stressors change function of the reproductive axis and pregnancy outcome (Brandt and Nielson, 1992; Ernst et al., 2015; Pawluski et al., 2015; Toufexis et al., 2014). Negative outcomes in

bright LL may not be due directly to circadian disturbance, but to continuing presence of environmental stressors.

A realistic acknowledgement of the advantages and limitations in each model of arrhythmia is of great importance in understanding reports of infertility in rodents with disrupted circadian function. Whether impaired fertility in these models of arrhythmia are due to a loss of time information, *per se*, or to effects of the manipulations that induce the loss of time information, is not clear. Each type of circadian disruption lacks a consistent response in reproductive outcome (See Section 1.7), strongly indicating a combination of features comprises circadian regulation of reproduction and fertility. No single methodology of inducing circadian disruption or arrhythmia is fully informative to the question. It is through the convergent inclusion of multiple models and integration of results that we will obtain a more complete understanding of mechanisms essential to circadian regulation of reproduction and fertility.

The purpose of the experiments in this chapter was to investigate the functional significance of the circadian clock and behavioral circadian rhythms in female reproductive function, using the DPS arrhythmic Siberian hamster as a model. The use of the DPS model of arrhythmia, in combination with previously studied models, furthers our current understanding of circadian regulation of reproduction. Initial experiments determined the level(s) of the hypothalamic-pituitary-gonadal (HPG) axis at which the loss of circadian timing information exerts its effects on the reproductive system. Specifically, these studies determined if plasma estradiol (Experiment 3.1) and LH surges (Experiment 3.2) were comparable in arrhythmic and entrained females across the estrous cycle. A series of experiments tested the hypothesis that arrhythmia-induced changes in the pattern of LH surges impairs fertility. This question was addressed via four mating studies: Acute 5-day cohabitation and long-term 90-day cohabitation

to determine reproductive success of a single litter and multiple, successive litters, respectively. 3-hour timed mate paring in either the active (Evening; ZT15-18) or inactive phase (Morning; ZT04-07) aimed to determine mating success at ecologically relevant times for this species. Each mating paradigm assessed multiple parameters of reproductive performance and fertility (Experiment 3.3).

3.2 Methods

Experiment 3.1: Plasma Estradiol

Disruptive Phase Shift: Hamsters (n=135; aged 2-4 months) were first housed for 4 weeks in a 16L:8D photoperiod, then subjected to the DPS protocol, as described in Section 2.2. 110 hamsters were subjected to the full DPS protocol. 25 hamsters were not subjected to the 2 h light pulse but received the 3 h phase-delay on the following day, a manipulation which, according to Ruby et al. (2004) does not lead to high rates of circadian arrhythmia. After DPS treatment, home-cage locomotor activity (LMA) was assessed as described in Section 2.3. Activity data were collected in a single 10 day interval occurring 4-8 weeks after the DPS treatment. A subset of females from this total DPS population was included in this study (4-6 months of age at time of experiment). Activity was continuously monitored to confirm maintenance of the ENTR/ARR behavioral chronotype throughout the duration of the experiment.

Plasma Estradiol Collection: In order to determine if circadian arrhythmia interferes with secretion of gonadal steroids required for ovulation, adult females (>6 months of age) were

individually housed in 16L:8D photoperiod, and classified as either entrained (ENTR; n=12) or arrhythmic (ARR; n=12) following DPS treatment. Unlike many rodent species, vaginal cytology is not a reliable indicator to determine estrous cyclicity in Siberian hamsters (McMillan and Wynne-Edwards, 1998; Dodge and Badura, 2002). Thus, to facilitate measurements of estradiol we attempted to induce synchrony of estrus via exposure to male cues, following methods described by Dodge et al. (2002): at ZT08 (12:00 CST) on Day 0 of the experiment, 6 ENTR and 6 ARR females were transferred into cages which had been previously inhabited by a gonadally-intact adult male (75-85 days old) for 7 days. The remaining 6 ENTR and 6 ARR females were transferred to cages with clean bedding. Females were then left undisturbed until the first blood collection, which occurred on the following day (Day 1). Males were removed from the cage immediately prior to introduction of the female.

Blood samples (250-350 μl) were obtained at ZT10 (14:00 CST) on 4 consecutive days, beginning on Day 1. Hamsters were lightly anesthetized with isoflurane, (3% in O_2) and blood was obtained from the right orbital sinus using a Natelson collection tube. Blood was transferred from the tube into a 1.5 ml eppendorf tube containing 15 μl heparin. Samples were immediately placed on ice until spun in centrifuge (700 x g**,** 25 min, 4 °C) to separate plasma. Plasma was transferred to a separate 1.5 ml eppendorf tubes and stored at -80 °C.

Estradiol EIA: Plasma estradiol was assayed in duplicate samples (diluted 1:2) via the 17β-Estradiol EIA kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturers instructions. Validation of the EIA kit was accomplished in part by inclusion of positive (plasma from cycling females) and negative (plasma from juvenile males) controls, with an inter assay variability of 13.9%, and a sensitivity of <17 pg/ml.

Experiment 3.2 Plasma LH

Disruptive Phase Shift: Hamsters (n=135; aged 2-4 months) were first housed for 4 weeks in a 16L:8D photoperiod, then subjected to the DPS protocol, as described in Section 2.2. 110 hamsters were subjected to the full DPS protocol. 25 hamsters were not subjected to the 2 h light pulse but received the 3 h phase-delay on the following day. After DPS treatment, home-cage LMA was assessed as described in Section 2.3. Activity data were collected in a single 10-day interval occurring 4-8 weeks after the DPS treatment. A subset of females from this total DPS population was included in this study (8-10 months of age at time of experiment). Activity was continuously monitored to confirm maintenance of the ENTR/ARR behavioral chronotype throughout the duration of the experiment.

Estradiol Implant Pilot Study: A pilot study was done in order to determine the size of subcutaneous estradiol implant, which would most accurately mimic endogenous levels of circulating estradiol. Uterine weights served as a bioassay for effective physiological estradiol treatments in ovariectomized hamsters. Entrained female hamsters (n= 30) were bilaterally ovariectomized (OVx) under isoflurane anesthesia (3% in O_2) and implanted with a subcutaneous capsule (2 mm, 4 mm or 8 mm silastic tubing; n=6 for each) containing β-estradiol (Sigma Chemical Co. St. Louis, MO). Control females were either sham operated (n=7) or OVx (n=6) and implanted with an 8 mm cholesterol capsule. All hamsters were administered standard post-operative buprenorphine analgesic (0.01 mg/kg) via subcutaneous injection (s.c.) 2×/day for 2 days after surgery and monitored for the duration of recovery. Body weight from all hamsters was monitored for three weeks following surgery, at which time hamsters were sacrificed and uterine tissue was collected and weighed.

Ovariectomy and Estradiol implants: In order to determine if the LH surge is present or disrupted in behaviorally arrhythmic hamsters, a subset of female hamsters (n=62) from the DPS population were identified as ENTR (n=33) or ARR (n=29) for this study. Females were then bilaterally ovariectomized (OVx) under isoflurane anesthesia (3% in O_2) and implanted with a subcutaneous capsule (0.5 mm, or 2 mm silastic tubing) containing β-estradiol (Sigma Chemical Co. St. Louis, MO), a standard procedure to induce a daily LH surge (Donham et al., 1987). ENTR and ARR control females were OVx and implanted with a 2 mm cholesterol capsule, which fails to induce an LH surge (Donham et al., 1987). All hamsters were administered standard post-operative buprenorphine analgesic (0.01 mg/kg, s.c.) 2×/day for 2 days after surgery and monitored for the duration of recovery.

Blood Collection: Following recovery from surgery (5 days), blood (250-350 µl) was obtained from all hamsters at 6 separate time points (ZT06, ZT11, ZT13, ZT14, ZT15, and ZT18), staggered over 4 days to allow for hemostasis and recovery from anesthesia as follows. On Day 1, blood was collected at ZT14. On Day 2, blood was collected at ZT13 and ZT18. On Day 3, blood was collected at ZT06 and ZT11. On Day 4, blood was collected at ZT15. For blood collections, hamsters were lightly anesthetized with isoflurane, (3% in O_2) and blood was obtained from the right orbital sinus using a 270 µl capillary tube. Hamsters were administered 0.5 ml sterile 0.9% saline (s.c.) for rehydration after each blood sample was taken. Blood was transferred from the capillary tube into a 1.5 ml eppendorf tube containing 15 µl heparin. Samples were immediately placed on ice until spun in centrifuge (700 x g**,** 25 min, 4 °C) to

separate plasma. Plasma was transferred to a separate 1.5 ml eppendorf tube and stored at -80 °C until time of assay. Blood samples were assayed via radioimmunoassy (RIA) as described below.

Radioimmunoassay: Plasma LH concentrations were determined in 10 to 100 µl aliquots. CSU 120 (100 µl; provided by Dr. Terry Nett, University of Colorado) was used as the primary antibody, diluted to 1:10,000 in normal rabbit serum 1:100 (Millipore, St. Charles, MO). Solutions remained in 0.05M PBS containing 0.1% gelatin. Samples were incubated for 48 hours at 4 °C following the addition of the primary antibody, and again following the addition of 100 µl radiolabeled LH (diluted to 22,000-32,000/100 µl cpm, MP Biomedicals LLC, Santa Ana, CA). A third 48 hour 4 °C incubation followed the addition of 200 µl secondary antibody (anti-rabbit gamma globulin, 1:50 in gel-PBS, Millipore, St. Charles, MO). All samples were analyzed in 3 assays for which the sensitivity averaged 0.025 ng/tube. Gamma emissions were counted in precipitate pellets in a Packard Cobra Gamma Counter for 1 minute per tube, in duplicate.

Experiment 3.3 Mating Behavior and Fertility Studies:

Expt 3.3.1. Acute 5-day pairing

Disruptive Phase Shift: Hamsters (n=82; aged 6-7 months) were first housed for 4 weeks in a 16L:8D photoperiod, then subjected to the DPS protocol, as described in Section 2.2. 62 hamsters were subjected to the full DPS protocol. 20 hamsters were not subjected to the 2 h light pulse but received the 3 h phase-delay on the following day. After DPS treatment, home-cage LMA was assessed as described in Section 2.3. Activity data were collected in a single 10 day interval occurring 4-8 weeks after the DPS treatment. A subset of females from this total DPS population was included in this study (8-10 months of age at time of experiment).

Fertility: Following DPS, adult female hamsters (n=30 ENTR, n=32 ARR) were each paired with an ENTR male for 5 days, encompassing a single estrous cycle. The day of pairing was defined as Day 0. All pairs were checked twice daily for the presence of a litter, and the number of pups in the litter was recorded. Pup checks were performed at ZT04 and ZT16. The day of litter birth was considered postnatal day (PND) 0. For each litter, the number of pups was counted on PND 0-2. After PND 2, dam and litter remained undisturbed until weaning on PND 18, with the exception of routine animal health care. Siberian hamster gestation is 18 days; day of mating, and latency to mate were inferred from the date of litter birth (Schum and Wynne-Edwards, 2005). At weaning, pups were sexed and weighed. The time of weaning remained consistent (ZT12) for the duration of the experiment. Latency to mate, number of pups at birth and weaning, pup mass at weaning, and litter sex ratio was recorded for each litter.

Expt 3.3.2. 90-day Cohabitation:

Disruptive Phase Shift: Hamsters (n=206; aged 3-5 months) were first housed for 4 weeks in a 16L:8D photoperiod, then subjected to the DPS protocol, as described in Section 2.2. 191 hamsters were subjected to the full DPS protocol. 15 hamsters were not subjected to the 2 h light pulse but received the 3 h phase-delay on the following day. After DPS treatment, home-cage LMA was assessed as described in Section 2.3. Activity data were collected in a single 10 day interval occurring 4-8 weeks after the DPS treatment. A subset of females from this total DPS population was included in this study (5-7 months of age at time of experiment). Activity was not monitored throughout the experiment, but dam and sire chronotype was confirmed within 4-6 weeks following weaning of final litter.

Fertility: Female hamsters (n=46) were paired males for 90 days, a standard paradigm for fertility testing in multiparous rodents (eg: Herbison et al., 2008). In this experiment, 4 permutations of circadian function were arranged as breeding pairs: ENTR female + ENTR male ("EE" pairing, n=12), ENTR female + ARR male ("EA" pairing, n=11), ARR female + ENTR male ("AE" pairing, n=12), and ARR female + ARR male ("AA" pairing, n=11). The day of pairing was defined as Day 0. All pairs (n=46) were checked 4 times daily for the presence of a litter, and the number of pups in the litter was counted. Pup checks were performed at ZT04, ZT08, ZT12, and ZT16 (8:00, 12:00, 16:00, and 20:00 CST, respectively). The date of litter birth was considered postnatal day (PND) 0. The number of pups was counted on PND 0-2. After PND 2, pairs remain undisturbed until weaning on PND 18. Latency to mate was inferred from the date of litter birth (Schum and Wynne-Edwards, 2005). At weaning, pups were sexed, weighed, and individually housed. The time of weaning remained consistent (ZT12) for the duration of the experiment. Latency to mate, inter-litter interval, number of pups at birth and weaning, pup mass at weaning, and number of litters were recorded for all pair combinations.

Expt 3.3.3. 3-h Timed Cohabitation

Disruptive Phase Shift (ZT15-ZT18 pairings): Hamsters (n=163; aged 4-5 months) were first housed for 4 weeks in a 16L:8D photoperiod, then subjected to the DPS protocol, as described in Section 2.2. 133 hamsters were subjected to the full DPS protocol. 30 hamsters were not subjected to the 2 h light pulse but received the 3 h phase-delay on the following day. After DPS treatment, home-cage LMA was assessed as described in Section 2.3. Activity data were collected in a single 10 day interval occurring 4-8 weeks after the DPS treatment. A subset of

females from this total DPS population was included in this study (6-7 months of age at time of breeding). Activity was monitored throughout the duration of the experiment.

Disruptive Phase Shift (ZT04-ZT07 pairings): Hamsters (n=211; aged 1-3 months) were first housed for 4 weeks in a 16L:8D photoperiod, then subjected to the DPS protocol, as described in Section 2.2. All hamsters were subjected to the full DPS protocol. After DPS treatment, home-cage LMA was assessed as described in Section 2.3. Activity data were collected in a single 10 day interval occurring 8-12 weeks after the DPS treatment. A subset of females from this total DPS population was included in this study (6-8 months of age at time of breeding). Activity was monitored throughout the duration of the experiment.

Mating Behavior: Female hamsters were paired with an ENTR male for 3 h at either ZT15-ZT18 (Evening; n=27 ENTR; n=23 ARR) or ZT04-ZT07 (Morning; n=25 ENTR; n=25 ARR) on 5 consecutive days. As with the acute pairing above, this time period encompassed an entire estrous cycle. ZT15-ZT18 pairings occur at a time when an ENTR hamster is in estrus (Fitzgerald and Zucker, 1976), but not necessarily an ARR female, based on results from Experiment 3.2. ZT04-ZT07 pairings occurred at a time when an ENTR animal was not likely to be in estrus, but an ARR female may be just as likely as in the ZT15-ZT18 paradigm. Mating behavior was recorded for the duration of all pairings. Mount number and latency, intromission number and latency, and ejaculation number and latency were recorded for each mating series. A completed series was defined as the progression of mount and intromission behaviors culminating in ejaculation. Total number of mating series, and interval between ejaculation and series onset were recorded. First day of pairing was considered Day 0.

Fertility: Following mating, all females were placed back on sensors and undisturbed until checks for litter births, aside from routine animal health care. Each dam was checked at ZT04 and ZT16 for presence of a litter from Days 17-23. The day of litter birth was considered postnatal day (PND) 0. For each litter, the number of pups was counted on PND 0-2. After PND 2, dam and litter remained undisturbed until weaning on PND 18, with the exception of routine animal health care. At weaning, pups were sexed and weighed. The time of weaning remained consistent (ZT12) for the duration of the experiment. Latency to mate, number of pups at birth and weaning, pup mass at weaning, and litter sex ratio was recorded for each litter.

Statistical Analyses

All locomotor activity analyses were performed as described in Chapter 2. Data were analyzed using the program StatView 5.0 (SAS Institute, Cary, NC, USA). Effects of circadian chronotype and estradiol were analyzed using repeated measures ANOVA. ANOVA (2[chronotype] × 2[Implant type] independent variables) was used to analyze amplitude of LH surge. Chi-square analysis was used to determine effect of circadian chronotype and presence of LH surge. Rayleigh Tests (Refinetti et al., 2007) were used to analyze significance in the circadian distribution of time for the LH surge. Chi-square was used to determine effect of chronotype in occurrence of mating behavior and litter birth. ANOVA was used to analyze circadian chronotype on reproductive and fertility measures in the acute and 3 h mating paradigms. ANOVA (2[dam chronotype] × 2[sire chronotype] independent variables) was performed for analysis of all measures of fertility in the 90-day cohabitation study. Pairwise comparisons were performed with two-tailed t-tests, only if a significant omnibus F-statistic was

obtained. A priori planned comparisons were performed without correction for family-wise error. Rayleigh tests were considered significant when $p<0.01$ (Refinetti et al., 2007). Chi-square, ANOVAs and pair-wise comparisons were considered significant when $p<0.05$.

3.3 Results:

Experiment 3.1: Plasma Estradiol

There was no significant effect of chronotype or exposure to male bedding on estradiol concentrations over the fours days of bleeds ($p>0.05$). Because no coherent social coordination of estrus was evident in females exposed to male bedding, we assumed that females were cycling independently of one another; thus, data from each female was arranged relative to the peak (highest estradiol value of the four days) value for each individual. The peak value was designated "peak"; the two preceding days were designated peak -1 and -2; and the day after the peak was designated peak +1. Such "sorting by peak" will, by definition, result in lower values at time points other than the defined peak, however, if the amplitude of the 4 day cycle generated via this approach differed between ARR and ENTR hamsters, then this would identify differences in ovarian hormone output between the chronotypes. Sorting data as such resulted in a significant pattern of plasma estradiol across the four days ($F_{3,30}=13.05$, $p>0.0001$), however the pattern did not differ between ARR and ENTR hamsters in its overall waveform ($F_{3,30}=0.96$, $p>0.05$; Fig 3.1). Moreover, collapsing across days, mean estradiol values did not differ between ARR and ENTR on any of the 4 sampling days ($p>0.05$).

Experiment 3.2: Plasma LH

Estradiol Pilot Study: Results from the pilot study determined in OVx+E females, there was a significant effect of implant size on uterine weight: larger implants resulted in heavier uteri ($F_{4,25}$=27.769, p<0.0001). The 2.0 mm implant yielded mean uterine weights greater than those of sham operated control animals (p<0.0001), which mimicked unmanipulated endogenous conditions. Constructing a best-fit line from these data (y=0.03x+0.134), it was determined via extrapolation that an implant size of 0.5 mm would generate a concentration of endogenous estradiol that would yield uterine weights approximately equal to those of un-operated females.

LH Surge: An LH surge was defined as a plasma LH value measuring 4 standard deviations above the overall mean LH value for the entire ENTR treatment group measured at ZT06 (a time of day when LH is not elevated; Legan et al., 2009). LH surges were abundant in females implanted with estradiol (64% of ENTR exhibited a surge, 52% of ARR exhibited a surge), but not in OVx females given cholesterol (0% of ENTR and ARR hamsters surged; χ^2= 8.24; p<0.01 vs. estradiol-implanted hamsters). In addition, ARR females were less likely to exhibit an LH surge in response to the smaller (0.5 mm) estradiol implant. There was a significant difference between the proportion of ARR females exhibiting an LH surge in response to a 0.5 mm implant, as compared to a 2.0 mm implant (χ^2= 3.83; p<0.05; Fig. 3.2). In contrast, an effect of implant size was not evident among ENTR females (χ^2= 0.84; p> 0.80; Fig. 3.2). The amplitude of the LH surge did not differ between behavioral chronotype groups ($F_{1,23}$=0.003, p>0.05) or implant size ($F_{1,23}$=1.1, p>0.05; Fig. 3.3).

Timing of the LH surge differed between ENTR and ARR females: A Rayleigh test indicated that ENTR females exhibited an LH surge which occurred with a significant temporal

precision, at a mean time of ZT11.4 ($R=0.98$, $p<0.001$; Fig. 3.4 A); in contrast, LH surges were more distributed in ARR females, and the phase was not significantly clustered in time to the same degree as that observed in ENTR hamsters ($R=0.57$, $p>0.01$; Fig. 3.4 B). Anecdotally, 2 ARR females, but no ENTR females, were recorded as having LH surges at two separate times (surge amplitudes at both ZT13 and ZT15 for each ARR female).

Experiment 3.3 Mating behavior and fertility

Expt 3.3.1 5-day Acute Cohabitation:

Reproductive performance between ENTR and ARR hamsters was relatively comparable. 63% of ENTR and 43% of ARR females successfully produced a litter, which did not significantly differ in a χ^2 analysis ($\chi^2= 0.32$; $p> 0.50$; Fig 3.5 A). Latency to insemination, measured by date of litter birth, was not different between the two groups ($p>0.1$). Interestingly, litter size was significantly larger for ENTR dams at birth, compared to ARR dams ($F_{1,35}=8.42$, $p<0.01$; Fig 3.5 B), and this result contributed to a larger litter at weaning ($F_{1,33}=5.26$, $p<0.03$; Fig 3.5 C). However, survival to weaning was not different between groups ($p>0.2$; Fig 3.5 D).

Expt 3.3.2 90-day Cohabitation

Analysis following cohabitation determined that 5 females (AA=2; AE=2; EA=1) and 8 males (AA=2; EA=6) had significantly changed chronotype over the duration of the experiment. Because the time of chronotype change could not be determined, data from these animals were excluded from analyses. Thus, sample size for each group was AA=6, AE=10, EA=4, and EE=12 in the following analyses. Over the 90-day cohabitation, almost all mate pairs (80% of AA, 90% AE, 100% EA, and 91.6% of EE) produced at least one litter. The latency to mate, interval

between litters, number of litters born, total number of pups born, total number of pups weaned, average size of litter at birth, average size of litter at weaning, or average percent of pup survival did not differ between groups ($F_{1,26}$<2.4, p>0.1 for all comparisons). Dam chronotype did not influence the aforementioned litter parameters, however, pup weight at weaning was significantly affected by maternal and paternal chronotype in both female (Dam: $F_{1,269}$=19.81, p<0.0001; Sire: $F_{1,269}$=13.85, p<0.0001; Fig 3.6 A) and male (Dam: $F_{1,347}$=4.51, p<0.04; Sire: $F_{1,347}$=18.8, p<0.0001; Fig 3.6 B) pups. For both male and female pups, this was characterized by decreased body weight in pups born to an ENTR dam and ARR sire (p<0.05). In female pups, there is also a decrease in body weight of pups with both ARR parents compared to pups with ARR dam and ENTR sire. Such an outcome suggests a paternally driven decrease in offspring body weight, in that pups with an ARR sire are smaller than those with an ENTR sire.

Expt 3.3.3 Acute 3-h Mating

For the ZT15-ZT18 (evening) pairings, analysis of activity data over the duration of the experiment shows that a total of 10 out of 50 females (ARR=9, ENTR=1) changed chronotype either following mating or in early gestation. In the ZT04-ZT07 (morning) pairings, analysis of activity data following weaning showed two out of 50 animals had changed chronotype (ARR=2) following mate pairing, or in early gestation. Because these females did not maintain a consistent chronotype for the duration of the study, they were excluded from analyses of mating behavior and fertility. Final sample sizes for the evening pairings were n=24 for ENTR and n=16 for ARR. For the morning pairings n=25 for ENTR and n=23 for ARR.

Mating behaviors: Breeding behavior between the morning and evening pairings showed distinct differences, which are characterized as follows. Of the animals paired for mating, 13/16 ARR and 19/24 ENTR (81.25% and 79.17%, respectively) demonstrated complete mating series in the evening. 10/23 ARR and 9/25 ENTR (43.48% and 36%, respectively) demonstrated complete mating series in the morning. Mating occurrence was lower in the morning compared to the evening in both ENTR (χ^2=9.32, p<0.003) and ARR (χ^2=5.56, p<0.02) hamsters (Fig 3.7 A). However, there were not any chronotype differences in mating occurrence in either the evening (χ^2= 0.03; p> 0.80) or the morning (χ^2= 0.12; p> 0.70; Fig 3.7 A). Onset to mating was significantly delayed in the evening compared to morning ($F_{1,57}$=5.77, p<0.02; Fig 3.7 B), but this did not differ between chronotype (p>0.4). Additionally, the number of completed series was significantly lower in the morning compared to the evening ($F_{1,84}$=10.12, p<0.003; Fig 3.7 C), but did not vary by chronotype (p>0.7).

Variance by time of day required further analysis in order to determine if these differences were due to circadian variation in male mating behavior, or in female mating behavior. In order to assess this question, we first looked at the total number of mounts by males, which showed a significant effect of time of day ($F_{1,84}$=6.97, p<0.01) a trend toward a main effect of chronotype ($F_{1,84}$=3.34, p>0.08; Fig 3.7 D). Unpaired comparisons showed that males mounted ENTR females significantly more during the evening vs. morning (p<0.006), but this effect was not observed among males paired with ARR females (p>0.23). Finally, there was a non-significant trend toward greater number of mounts in ARR females in the morning compared to ENTR females (p<0.1). Thus, male mounting behavior showed significant circadian variation in the presence of an ENTR female but not when paired with ARR females.

In females, a measure of sexually receptive behavior could be assessed via the number of mounts in which she permitted the male to intromit. Note that this measure is not isomorphic with lordosis, as we did not specifically quantify lordosis behavior during behavioral analyses (i.e., male mountings that did not culminate in an intromission, which were plentiful, may nevertheless have elicited a lordosis reflex in females, however, the experimenter's eyes were trained on the male during behavioral observations in order to observe for thrusting behavior. This analysis showed no effect of time of day or chronotype ($p>0.12$ for both), suggesting that females of both chronotypes were equally likely to permit intromission at both times of day (Fig 3.7 E).

Fertility: time of day did not affect pregnancy rates. The proportion of females that permitted a full copulatory cycle and eventually delivered a litter did not differ by time of day in ENTR (morning vs. evening: $\chi^2=3.33$, $p>0.0.6$) or ARR (morning vs. evening: $\chi^2=1.81$, $p>0.17$) hamsters (Fig 3.8 A). A non-significant trend of time of day in litter size at birth ($F_{1,30}=2.93$, $p<0.1$; Fig 3.8 B) was characterized by smaller litter sizes in ENTR female mating in the morning. Interestingly, the effect size for time of day on litter size, measured by Cohen's *d'*, was almost twice as large in ENTR females compared to ARR females (0.78 and 0.47, respectively). Number of pups at weaning, and the percent of pups surviving to weaning did not differ between time of day or chronotype ($p>0.3$ for all comparisons). At weaning, female pups showed no effect of chronotype or time of day in body weight ($p>0.2$; Fig 3.8 C). A significant effect of time of day in male pups ($F_{1,56}=7.67$, $p<0.008$; Fig 3.8 D), was characterized by lower body weight in pups born to evening mated dams compared to morning mated dams. There was no effect of chronotype for male pups ($p>0.2$).

3.4 Discussion

ARR females maintained a coherent 4-day estrous cycle, which was also characterized by a peak amplitude of estradiol comparable to that of ENTR females. In an OVx+E model to induce a daily LH surge, unlike ENTR females, ARR females did not exhibit robust circadian rhythm in the LH surge: LH surges were concentrated in a 2 h window of time in ENTR females, but were distributed across 12 h of the day in ARR females. ARR females also required a higher concentration of estradiol in order to trigger an LH surge, as compared to ENTR females. However, the amplitude of the LH surge in ARR females did not differ from that of ENTR females. Together, these data suggest that behaviorally arrhythmic hamsters are still capable of exhibiting LH surges, but these surges are not locked to a circadian time, as is the case in ENTR females. The circadian 'gate' for LH surges is markedly diminished if not absent in ARR females.

Despite these underlying impairments in reproductive neuroendocrine function, major aspects of fertility and reproductive output were qualitatively normal. Specifically, acute cohabitation showed smaller litter sizes, but normal survival. Long-term cohabitation demonstrated all measures of fertility to be indistinguishable across circadian chronotype. Acute timed mating in the morning and evening revealed circadian rhythms in mating behavior (see below), but these were driven by the behavior of the ENTR stud male, and no differences between time of day, or chronotype were evident in in measures of fertility. Thus the data indicate that circadian timing information is critical to maintenance of a normal phase for LH surges and may impact hypothalamic/pituitary sensitivity to estradiol positive feedback; however, these effects of the circadian system, when absent in the ARR hamster, do not translate

into any substantial compromise in fertility. Taken together, these experiments create a picture of a temporally disrupted, but overall functional reproductive system in female hamsters, even in the face of behavioral arrhythmia.

Although lacking a functional SCN, ARR females maintained a coherent 4-day infradian rhythm of estradiol, comparable to that of an ENTR female. Mean concentrations of estradiol were also comparable to those of ENTR females. These data indicate that, despite behavioral arrhythmia, and coherent output from the SCN, one critical aspect of gonadal hormone secretion is qualitatively normal in the ARR female. It is remarkable that the approximate 4-day timing of the estradiol peak remains intact in ARR females. This outcome suggests that some other source of time information remains intact and can provide temporal order to ovarian hormone release. This may reflect non-circadian infradian rhythms in ovarian estradiol production. However, peripheral circadian clocks may participate in this phenomenon as well. Oscillations in ovarian clock gene expression have been identified (Fahrenhrug et al. 2006; Karman and Tischkau, 2006), and functionally implicated in the timing of ovulation (Selix et al. 2010). Mice homogenous for the *clock* mutation exhibit significant irregularities in the period of the estrous cycle, lack a proestrus LH surge, and exhibit increased fetal reabsorption (Miller et al., 2004). However, circulating concentrations of estradiol in *clock* mutants were comparable to those of wild-type mice, and follicular development in the ovary was not disrupted: follicles at all stages of development were present, and in comparable numbers to that observed in wild-type tissue (Miller et al. 2004). This suggests that reproductive deficits due to clock-gene mutation do not impact function at the level of the ovary. The present data are consistent with such an observation. An intact 4-day rhythm, and normal timing of the estradiol peak at ZT10, suggests that the ovary of the ARR hamster is capable of exhibiting essentially normal 4 day cycles,

despite the absence of a functional circadian pacemaker in the CNS. Circadian rhythms in ovarian function may be resistant to decay/dampening when disconnected from the pacemaker in the SCN, or perhaps more likely, the 4 day cycle in ovarian steroid production may not depend on circadian processes in the brain or ovary itself.

In contrast to persistent rhythms in estradiol, LH surges lacked circadian synchrony. LH surges occurred in ARR females without significant circadian clustering, in marked contrast to the temporal clustering observed in ENTR females. ARR females evidently lack the circadian "gate" necessary to phase the LH surge, as the current model of the rodent estrous cycle holds (Fitzgerald & Zucker, 1976; Meyer-Bernstein et al. 1999). However, ARR females did exhibit LH surges, and despite a lack of coordination in the timing of the LH surge, the amplitude of the LH surge was normal as compared to an ENTR female. Thus, once generated, the LH surge of an ARR female is quantitatively comparable to that of an ENTR female.

Arrhythmia had a substantial impact on sensitivity to estradiol. The estradiol threshold for the induction of an LH surge is elevated in the ARR female; a smaller proportion of ARR females had a surge with a 0.5 mm implant than with a 2 mm implant. Small, 0.5 mm implants were highly effective in inducing LH surges in ENTR females; thus, the ARR female required a higher concentration of endogenous estradiol in order to achieve an LH surge. Such a result may initially appear to be due to the spread of surge time in ARR hamsters. However, the proportion of surges for ARR hamsters with the 2 mm implant (who are equally vulnerable to spread in surge time) did not differ from that of ENTR hamsters with an implant of either size. Thus, a decrease in sensitivity to estradiol in ARR females appears the most plausible hypothesis to explain this outcome.

Based on the current understanding of the requirements for a proestrus LH surge in the rodent, it is remarkable that ARR females have an LH surge at all, and more so that it is of normal amplitude. ARR females should entirely lack the SCN "gating" signal, which is considered mandatory in order to produce an LH surge (Kriegsfield and Silver, 2006). In light of this, the role of hypothalamic RF-amide neuronal populations must be further considered in an examination of the neural mechanisms by which circadian arrhythmia disrupts reproduction and fertility. Whereas RFRP-3 has been considered the mediator of negative feedback (see Section 1.6), a population of cells in the anteroventral periventricular nucleus (AVPV) of the hypothalamus, Kisspeptin-containing cells, seem to be the key mediator of positive feedback in the neural regulation of reproductive function. Kisspeptin (Kiss1*)* neurons in the AVPV have been strongly implicated as a key component of the neural pathway that triggers a circadian LH surge (de la Iglesia and Schwartz, 2006; Kinoshita et al., 2005; Smith et al., 2006). Kiss1 neurons do express ERα, and also receive direct neural input from the SCN (Robertson et al., 2009). Projections from the dorsalmedial SCN (dmSCN) have been shown to project onto Kiss1 neurons in the AVPV, and to influence timing of the LH surge, independent of the light-dark cycle (Smarr, Morris, and de la Iglesia, 2012). Recent evidence also supports the presence of an estrogen-dependent peripheral clock in the AVPV Kiss1 population, such that sensitivity to circadian signaling is gated by the presence of estradiol, and timed by internal oscillations (Chassard et al., 2015; Smarr et al., 2013). Any oscillatory function retained by the dmSCN following DPS-induced arrhythmia may be sufficient to maintain timing of the peripheral clock in the AVPV, and subsequently the LH surge.

Alternatively, ARR females may achieve an LH surge in a similar fashion to an induced ovulator. In induced ovulation, an exogenous stimulus, typically the act of mating, and not

circadian control is required to induce the LH surge and ovulation, (Exley et al. 1968; McCarthy and Becker 2002). In the present study (Experiments 3.1 and 3.2), ARR females remained socially isolated, and were not exposed to any conspecifics in the home cage (which might have been able to trigger LH surge). However, it is possible that, with the correct concentration of endogenous estradiol, a certain threshold is reached to override the circadian "gate" in the ARR female. A caveat should be added that LH surges were being elicited using constant release implants of estradiol, which likely do not mimic normal daily fluctuations in this hormone, and therefore, insights gained from the OVx+E model may not directly address the issue of whether hamsters, in their unmanipulated state, are induced vs. spontaneous ovulators. Nevertheless, one possibility is that, following induction of arrhythmia, reproductive function could still be maintained given a high enough concentration of estradiol to induce ovulation. The LH surge in ARR females occurs regardless of the time of day, and is not reduced in amplitude, further supporting this possible mechanism of the LH surge function outside of circadian control. The presence of induced components of ovulation in typically spontaneous species has been identified in the Djungarian hamster (*Phodopus campbelli*), characterized by an increase in mating on the third day after pairing (Erb et al., 1993). This phenomenon was proposed in Siberian hamsters (Dodge et al., 2002), but not observed in any of the mating paradigms tested here (but note caveat above). Plasma LH in males increased via exposure to a female in mice (Coquelin et al. 1984), sheep (Sanford and Yarney, 1983), and Siberian hamsters (Anand et al. 2002). If the reverse is true, the presence of a male in the following experiments may have been sufficient to elevate estradiol, and thus permit LH surges and relatively normal fertility in ARR female hamsters.

Behaviorally arrhythmic hamsters paired with an entrained male for 5 days showed normal fertility, in terms of the proportion of females becoming pregnant and the latency to mating. ARR females had smaller litters than ENTR females, an effect which was not observed in any other paradigm. At weaning, both pup survival and body weight was comparable between chronotypes. In this paradigm, ARR females showed somewhat reduced offspring output, but relatively normal reproductive rates and, presumably, maternal care. In contrast with the acute pairing, behaviorally arrhythmic female hamsters did not exhibit any major decrements in fertility during a 90-day cohabitation with either an ENTR or ARR male. The latency to mating, interval between litter births, number of litters, number of pups at birth and weaning was comparable between breeding pair types, regardless of behavioral chronotype. Curiously, in the 90-day cohabitation, both male and female pups showed a reduction in body weight when the sire was ARR. Reduction in mean pup body weight was not accompanied by reduction in pup survival to weaning. However, this suggests a role of paternal chronotype in either food intake or growth rate in Siberian hamster pups. Siberian hamsters are not a bi-parental species, although the presence of a male does not have a negative impact on pup survival and condition (Newkirk et al., 1998; Timonin and Wynne-Edwards, 2006; Wynne-Edwards and Lisk, 1989). While the male is presumably not contributing to the general survival of the pups, presence of a male in the home cage may alter conditions of food availability or thermoregulation to the pups via disrupted access to the dam. The arrhythmic nature of these environmental changes would result in higher energetic demands for litters with ARR sires, and decreased body weight.

Inclusion of the ARR male in the design of this study permitted an analysis of possible social interactions between dam and sire as a non-photic *zeitgeber* for the ARR female. It is well established that timing of social interaction can act to shift the clock, dependent on when social

interaction occurs (Cambras et al., 2012; Goel and Lee, 1997; Mrosovsky, 1988). In this study, measures of reproductive output were comparable in ARR females, regardless of male chronotype. If a social mechanism of time communication were responsible for maintenance of fertility in the ARR female, then one would predict impaired reproductive performance in ARR females when paired with an ARR male, but this was not the case. The ENTR male is evidently not communicating meaningful or additional circadian information to the ARR female, at least not at a threshold necessary to meaningfully stimulate changes in chronotype, which may depend on group size (Paul et al., 2014; Paul et al., 2015). Results of these data also indicate that the ARR male was not reproductively compromised. However, it is worth noting that switching of chronotypes in females over the duration of the study was equally distributed across groups. In the males, a change from the ARR to ENTR chronotype was predominant when paired with an ENTR female. While speculative based on current data, it is possible that the circadian social interaction afforded by a single ENTR cagemate may be more strongly impacting the circadian system of ARR males, than ARR females.

The timed, 3-hour mating studies were designed to determine rhythmicity in copulatory behavior, and time of mating influence on subsequent litter birth and viability. Mating behavior was significantly more prominent in hamsters paired in the evening (active phase) compared to the morning (inactive phase), but not different between female ARR or ENTR chronotype. Examination of the data determined that it was male-driven, proceptive behavior, which was varied over the circadian cycle. Such a result is not entirely unexpected, based on mating rhythms found in nocturnal male Syrian hamsters (*Mesocricetus auratus*; Eskes, 1984), Sprague-Dawley rats (*Rattus norvegicus*; Landry et al., 2012; Harlan et al., 1980), and in diurnal grass rats (*Arvicanthis niloticus*; Mahoney and Smale, 2005). All of these species exhibit mating

behaviors and frequencies peaking in the active phase. In contrast to male rhythms, measures of female receptive behaviors (as inferred only by intromissions allowed, as absolute numbers of lordosis events were not measured) did not vary between time of day or chronotype. This was also consistent with existing data, showing that steroid-primed females in multiple rodent species maintain relatively invariant mating behaviors over the course of the day (Erskine et al., 1980; Eskes, 1984; Harlan et al., 1980; Mahoney and Smale, 2005). Changes in sex behavior likely reflected an interaction between an ENTR male's response to an ARR vs. an ENTR female. The data indicate that ARR females elicited comparable levels of male proceptive behavior regardless of time of day, in contrast to ENTR females (Fig. 3.7). Although untested here, one would predict that the daily rhythm evident in mounting exhibited by ENTR males interacting with ENTR females would be absent in ARR males interacting with ARR females; but whether, in this dyadic interaction of differing chronotypes, an ENTR female would be capable of inducing a CR in mounting behavior in an ARR male remains an open question, the answer to which would inform us as to the relative importance of the male and female chronotype in the generation of daily rhythms in sex behavior.

Given the essentially random nature of LH surges in ARR females, we predicted that the morning vs. evening mating paradigm would not affect fertility rates in ARR females but would impact them in ENTR females. Despite lower incidence of mating in the morning compared to the evening (likely driven by behavior of the ENTR stud male, as discussed above), the proportion of females giving birth to a litter did not differ between chronotype or time of day. All measures of fertility were comparable between chronotype and time, with the exception of a trend towards smaller litter sizes at birth in ENTR females mated in the morning, while ARR females remained consistent across times of day. Following ovulation, unfertilized oocytes

remain in the oviduct (Takahashi et al., 2013). The optimal window for fertilization and embryonic development in Syrian hamsters and mice is less than 10 hours (Sakai and Endo, 1988; Yanagimachi and Chang, 1961). Because ENTR females maintain timing of the LH surge, they may have a longer duration between ovulation and the onset of morning pairing, compared to the randomly cycling ARR female. Degradation of postovulatory oocyte quality over time can result in fewer fertilized eggs and viable embryos, and thus a smaller litter size (Lord et al., 2013; Tarin et al., 1999; Wang and Sun, 2007). While speculative based on the current data, the observation of a non-significant trend (accompanied by a strong *d'*) towards smaller litter sizes in morning-mated ENTR females (and the absence of any semblance of a time-of-day effect in this measure in ARR females) is consistent with the conjecture that greater degradation of oocyte quality may have occurred between ovulation and mating in ENTR females between evening and morning, whereas such an effect may not impact presumably randomly-ovulating ARR females.

In the ARR female hamster, disruption in the timing of the LH surge did not translate into decreased fertility. The reproductive system is evidently able to compensate for a lack of circadian entrainment. One mechanism, discussed above, by which this may occur, is via reverting to a mechanism of induced ovulation. Alternatively, females may simply show persistent desynchrony in ovulation, perhaps mediated by continued estrogen-dependent oscillation of RF-amide neuronal populations, and rely on the male to be available when she does ovulate. Due to rhythmicity in male mating behavior, this may not be a viable strategy in a naturalistic setting, absent controlled, laboratory conditions. While ARR females are capable of producing an LH surge and maintaining pregnancy, a behaviorally arrhythmic female subjected to a less contrived social setting would be unlikely to have the opportunity to mate at the time of her LH surge.

In summary, the present data indicate that ARR female hamsters exhibited normal estradiol, disrupted phase of LH surges, and elevated thresholds for steroid induction of LH compared to ENTR females. This outcome is consistent with other models (lesions, germ line clock gene mutations) of arrhythmia, in which LH surges are disrupted, and extend the reproductive consequences of circadian disruption to include changes in responsiveness to steroid hormone positive feedback. Thus, in this regard, the data support the lesion/mutation approach, suggesting that reproductive neuroendocrine deficits observed in lesion/mutation models of arrhythmia are not merely side-effects of the methods by which arrhythmia is induced, but rather may indeed reflect the loss of circadian time information.

Most major aspects of fertility, however, were unimpaired in ARR females, regardless of mating paradigm. So in this respect, the data indicate that a loss of time information, *per se*, cannot account for reproductive impairments in this model, as few were seen. Other circadian disruption models exhibit impaired fertility (reviewed above), and a question remains whether the loss of time information is the primary causative factor; however, we acknowledge that many differences exist between the DPS arrhythmic hamster and the multiple other models of arrhythmia. Nevertheless, using a non-invasive method of inducing behavioral arrhythmia, this study reports disruption in timing of the LH surge, but that rhythms in ovarian estradiol and overall fertility are preserved absent a functional circadian clock. Fertility in the DPS hamster is resilient to behavioral arrhythmia. Taken together, these experiments provide novel insights into our current understanding of circadian influence of the reproductive system.

Chapter 4: Circadian effects on wound healing in female Siberian hamsters

4.1 Introduction

Immune activity is under direct control of the circadian system via the suprachiasmatic nucleus (SCN) of the hypothalamus. Output from the SCN drives daily variation in multiple aspects of adaptive and innate immune function: Leukocytes are trafficked from the circulatory system to peripheral tissues in a circadian pattern, with concentrations of blood leukocytes high during the inactive period, and low during the active period (Dahbhar et al., 1994). Cytokine production fluctuates with a circadian rhythm, in vivo and in vitro (Esquifino et al., 2007), thus altering the capacity to respond to immune challenge based on time of day. Severity of infection-induced acute inflammatory response and subsequent expression of sickness behaviors is dependent on the time of day (Arjona and Sarker, 2005; Arjona and Sarker, 2006; Guan et al., 2005; Marpegan et al., 2005). Mice have a more severe response to simulated bacterial infection via injection of lippopolysaccaride (LPS), if injected in the late rest phase compared to the mid-active phase (Franklin et al., 2003; Marpegan et al., 2009). A measure of adaptive immune response, inflammation following delayed-type hypersensitivity (DTH) is most apparent when exposure occurs in the rest phase compared to the active phase (Pownall et al., 1979).

The necessity of stable circadian regulation of the immune system is remarkably apparent when normal circadian entrainment is disrupted or eliminated. Individuals engaging in extensive transmaridian travel, night-time or rotating shift work are diagnosed with various cancers at a higher rate (Pukkala et al., 2002; Reynolds et al., 2002; Schernhammer et al., 2001). Stable circadian entrainment increases quality of life and survival in cancer patients (Innominato et al., 2012; Mormont et al., 2000; Sephton et al., 2000). Repeated phase shifts in mice disrupt

circadian function and exponentially increase mortality following LPS injection (Castanon-Cervantes et al., 2010). Dim light during the dark phase is sufficient to suppress both the adaptive and innate immune response in Siberian hamsters (Bedrosian et al., 2011). Complete behavioral arrhythmia eliminates normal leukocyte trafficking in Siberian hamsters, resulting in constantly elevated blood leukocyte concentration compared to the daily peak and trough seen in entrained animals (Prendergast et al., 2013). Considering the importance of rapid leukocyte response following immune challenge or tissue damage (Mercado et al., 2002; Rojas et al., 2002), a lack of coordinated trafficking from blood to tissues may have considerable negative consequences in the organism. Coordination of the immune system via circadian influence is essential for normal immunocompetence.

Initial inflammatory response, proliferation, and tissue remodeling constitute the extensive immunological processes that result following damage to cutaneous tissue (Guo and DiPietro, 2010)**.** Leukocyte deployment is essential for a rapid response to a skin injury (typically a wound); leukocyte infiltration follows the initial inflammatory phase of wound healing and persists for several days thereafter (Velnar et al., 2009), and contributes to tissue repair and remodeling. Acute and chronic stressors have been shown to slow healing following a wound in many species (Godbout and Glaser, 2006; Kiecolt-Glaser et al., 1995; Marucha et al., 1998). Wounds administered following restraint stress in rats show reduced initial recruitment of pro-inflammatory cytokines, including KGF-1, and IL-1b, suggesting an alteration of inflammatory kinetics following physical restraint stress (Mercado et al., 2002). In Siberian hamsters, the social environment plays a role in the speed of wound healing, with group-housed animals recovering faster than isolates with the same wound (Detillion et al., 2004). Such evidence supports the idea that coordination of tissue repair and remodeling is subject to both

positive and negative influence of physiological function outside of the immune system. However, cutaneous wound healing has yet to be assessed as a process under the influence of the circadian system.

In order to develop a more complete understanding of both the behavioral and physiological influences of circadian disruption on wound healing, we opted to utilize the DPS methodology of inducing arrhythmia in Siberian hamsters. The delivery of a precise combination of nocturnal light treatments permanently eliminates circadian rhythms in sleep/wake, body temperature, and activity (Ruby et al. 1996; Ruby, Barakat & Heller 2004). These light treatments also render SCN clock gene expression (*per1*, *per2*, *bmal1*, and *cry1*) arrhythmic via amplitude suppression of the circadian pacemaker (Grone et al. 2011). Glial scarring following SCN lesion (Logan et al., 1992; Silver and Miller, 2004), pleiotropic effects of clock gene mutation (Bur et al., 2009; Male et al., 2012), and chronic stress induced by bright light (Daan and Pittendrigh, 1976; Welberg et al., 2006) all limit the interpretation of immunological measures in traditional models of arrhythmia. The DPS method has the advantage of allowing animals to remain undisturbed in a standard light-dark cycle, without traditional confounds. Using this neurologically- and genetically-intact model of circadian arrhythmia, it is possible to investigate the functional consequences of complete circadian desynchrony in the immune system. This approach allowed us to gain insights into whether immune deficiencies observed following classical methods of arrhythmia induction are a cause of the loss of time information per se, or merely a side effect of the method by which the animal was rendered arrhythmic.

The purpose of this experiment was to examine the functional relevance of coherent circadian rhythms in the immune system towards organismal-level aspects of immune function. We hypothesized (1) that CRs in wound healing may exist, and (2) that in the absence of the

coordinated rhythm in trafficking of white blood cells (in arrhythmic hamsters), either CRs in wound healing would be eliminated, or wound healing would be impaired (or both). These experiments thus tested the hypothesis that circadian control of the immune system influences the healing rate of a cutaneous wound. Entrained and arrhythmic females, single-housed in a 16L:8D photoperiod, received a cutaneous wound either in the early light phase (ZT03) or early dark phase (ZT18). The area of the wound and the rate of healing were calculated via daily photographs over the following 17 days. Outcome of these data will elucidate the role of circadian organization in coordinating the temporal order of mechanisms underlying cutaneous wound healing, including inflammation, leukocyte trafficking, and tissue remodeling, and possibly through behavioral mechanisms.

4.2 Methods

Animals: Female Siberian hamsters (*Phodopus sungorus*) were derived from a breeding colony maintained in a long-day, 15L:9D photoperiod (LD) at the University of Chicago. Hamsters were housed in polypropylene cages, with food (Harlan, Teklad) and filtered tap water provided *ad libitum*; cotton nesting material was available in the cages. Ambient temperature and relative humidity were held constant at 19 ±2°C and 53 ±10%, respectively. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Chicago. Hamsters were subjected to the circadian disruptive phase shift procedure (DPS; see below) at 2-4 months of age.

Circadian rhythm disruption (DPS procedure): The DPS manipulation that destabilizes the hamster circadian pacemaker employs phase-resetting light stimuli that render a substantial

proportion of hamsters behaviorally circadian arrhythmic ("ARR"; Ruby et al., 2004) and disrupts SCN clock gene expression (Grone et al., 2011). Hamsters for this experiment were drawn from a larger cohort (n=206) of female hamsters that were subjected to the DPS procedure and then were assigned to different experiments. For the DPS procedure hamsters, were first housed for 4 weeks in a 16L:8D photoperiod, then on a single night, a 2 h light pulse was administered during the 5th through 7th h of the dark phase. The next day, the 16L:8D photocycle was phase-delayed by 3 h, by extending the light phase (as described in Section 2.2). Of the hamsters selected for the present study, 35 were subjected to the DPS protocol which included the 2 h nocturnal light pulse; 8 hamsters were not subjected to the 2 h light pulse but received the 3 h phase-delay on the following day, a manipulation which does not lead to high rates of circadian arrhythmia (Ruby et al., 2004).

After DPS treatment, home-cage locomotor activity (LMA) was assessed using passive infrared motion detectors positioned outside the cage (22 cm above the cage floor). Motion detectors registered activity when 3 of 27 zones were crossed. Activity triggered closure of an electronic relay recorded by a computer running ClockLab software (Actimetrics, Evanston, IL). Cumulative activity counts were collected at 1 min intervals. Activity data for circadian chronotyping (see below) were collected in a single 10 day interval occurring 10-11 months after the DPS treatment, and 12-30 days before cutaneous wound healing was assessed (cf. Ruby et al., 1998).

Circadian Chronotyping following DPS: Criteria for assessing the presence/absence of CRs were similar to those in prior reports of DPS-induced CR disruption (Ruby et al., 2004; Ruby et al., 1998). χ^2 periodogram and Lomb-Scargle periodogram (LSPs) were used to detect

presence/absence of CRs. LSP analyses were implemented to compliment χ^2 analyses, due to the increased sensitivity of the LSP in detecting CRs in non-sinusoidal data (e.g., 'square-wave' data, typical of circadian-entrained hamsters) and an increased propensity for χ^2 periodograms to indicate false peaks in noisy data (as is typical of DPS-induced ARR hamsters; Ruf, 1999; Refinetti et al., 2007). χ^2 and LSP analyses were performed on a 10 day block of activity data (cf. Ruby et al., 1998). CR amplitude was quantified in all hamsters via Qp values at 24.0 h from the χ^2 periodogram.

Hamsters were designated as entrained (ENTR) if they exhibited: (1) significant ($P<0.001$) circadian ($22 \text{ h} < \text{tau} < 26 \text{ h}$) activity peaks in both the χ^2 periodogram and the LSP, (2) consistently clear daily activity onsets and offsets upon visual inspection of the actogram, and (3) activity predominantly restricted to the dark phase of the LD cycle.

Hamsters were designated as arrhythmic (ARR) if they exhibited: (1) the absence of clear and significant ($P<0.001$) circadian peaks in χ^2 periodogram or the LSP, (2) an absence of consistently clear daily activity onsets and offsets, and (3) LMA distributed throughout the light and dark phases of the LD cycle.

Three hamsters exhibited activity patterns that were not easily classifiable. One hamster exhibited a temporally-narrow (<1.8 min bandwidth) 'spike' in the χ^2 periodogram which did not resemble a normal circadian peak, but nevertheless exceeded the χ^2 significance level of $P<0.0001$, indicative of power at a restricted frequency. The χ^2 spike exhibited no significant power in the surrounding temporal domain (atypical of hamsters with normal CRs) and was 8.9 Qp units above the significance level; cf. a mean of 269.0 Qp units above significance level in ENTR hamsters). This animal also lacked clear circadian activity onsets and offsets, exhibited activity throughout the day and night, and exhibited almost no detectable power in the LSP (PN

= 0.21) or the FFT (relative power = 0.001). For these reasons the hamster was classified as ARR. A second hamster exhibited modest but significant peaks in the χ^2 periodogram and the LSP, but lacked clear and consistent activity onsets and offsets, and exhibited locomotor activity throughout the light and dark phases on 4 of 10 assessment days. A third hamster, although lacking significant peaks in the χ^2 periodogram and LSP, exhibited a free-running activity pattern (tau ~ 25 h) with clear onsets and offsets on 7-8 of the assessment days. Due to their ambiguous and rhythmic chronotypes, respectively, these latter two hamsters were excluded from analyses. 26 ENTR and 14 ARR hamsters exhibited clear chronotypes and were used in these experiments.

Skin wounding procedure: Hamsters were administered a circular cutaneous wound using a 3.5 mm punch biopsy tool according to methods detailed elsewhere (Kinsey et al., 2003). Briefly, hamsters were lightly anesthetized with isoflurane, and a patch of fur approximately 90 mm^2 on the dorsal surface was shaved with electric clippers. The shaved region was sterilized using Betadine solution (Purdue Fredrick, Stamford, CT) and two uniform, circular wounds 3.5 mm in diameter were made simultaneously in the dorsal skin using a sterile, disposable punch biopsy tool (Manufacturer, Location). Wounds (entrance and exit) were bilateral, but only data generated from analyses of the entrance wound were used in statistical analyses (Kiecolt-Glaser et al., 1995).

Immediately following wounding ("Day 0"), and at 24 h intervals thereafter for the next 17 days, each wound was photographed using a digital camera (Canon Powershot ELPH110HS, Melville, NY); a reference standard (a printed black 3 mm diameter circle on a white paper background) was included in every photograph. In each digital image, entrance wounds and reference standards were traced at 150× magnification, and their respective areas were calculated

using graphic design software (Adobe Illustrator). The area of each wound was divided by the area of the reference standard in each photograph; the resultant ratio was provided a measure of standardized wound size (SWS) for each wound (Kiecolt-Glaser et al., 1995).

All skin wounding was performed by the same experimenter, as was all subsequent skin photography and image analysis. In order to consolidate skin wounding and daily skin photography into the specified 1 hour circadian time interval, skin wounding was performed in two separate rounds, separated by 20 days. In Round 1, 16 ENTR hamsters were randomly assigned to receive skin wounds at either ZT03 (n=8) or ZT18 (n=8) in order to determine if CRs in skin wounding manifest. ENTR hamsters with the subjectively most robust entrained actograms were included in Round 1.

In the second round of wounding (Round 2), the remaining 10 ENTR hamsters (ZT03: n=5; ZT18: n=5), and 14 ARR (ZT03: n=8; ZT18: n=6) hamsters received skin wounds. Hamsters were randomly assigned to ZT groups, and all wounding procedures and analyses were performed blind to chronotype.

Wound healing metrics: Percent change in wound size for each animal was calculated on each day as the ratio of SWS of the wound on a given day to the SWS of the same individual's wound on Day 0, thus providing a measure of relative wound size (RWS). Wounds were considered 50% healed on the first day that wounds were 50% reduced in size from day 0 values and remained so upon all measurements thereafter. Analogous calculations were performed to determine the day on which wounds were 100% healed. A minority of hamsters (n=2) did not achieve the criteria for 100% healing by day 17 and were assigned a default value of day 17 for 100% healing.

Validation of wound assessments during the dark and light phases: In pilot studies, we determined that the focal length of the digital camera would vary when photographs were taken under dim red light (at ZT18) as compared to under normal room illumination (at ZT03). In order to ensure that wounds were being comparably measured when photographs were taken in the dark and light phases, we performed a validation experiment. In this pilot study (n=6), dorsal cutaneous wounds were performed and immediately photographed. Wounds were performed under normal room illumination (during the light phase). Wounds were first photographed under normal room illumination, and immediately thereafter the lights were turned off and wounds were photographed again under dim red light. For the purposes of increasing sample sizes for this validation procedure, both entrance and exit wound sizes were calculated relative to the reference standard, and thus SWS values were calculated for a total of 12 wounds. There was a significant linear correlation between SWS values for the same 12 wound measurements obtained during the L and the D phase (R^2=0.98, P<0.0001), indicating that wounds could be precisely and accurately measured under both conditions of illumination.

Locomotor activity analyses: Relations between patterns of locomotor activity during the interval shortly following wounding and rates of wound healing were also assessed. To facilitate photography, hamsters were not housed under passive infrared monitoring lids post-wounding, therefore, assessments of activity were obtained from activity records generated 12-30 days prior to wounding (i.e., locomotor activity files used for circadian chronotyping). Mean total activity counts (over 10 days) were quantified during two separate 8 hour intervals: ZT03-ZT11 (for

ZT03 wounded hamsters) and ZT18-ZT02 (for ZT18 wounded hamsters), corresponding to the 8 h following ZT03 and ZT18 wounds, respectively.

Statistical analyses: CR amplitude measures (χ^2 periodogram, Lomb-Scargle periodogram and FFT values) were compared using t-tests. Standardized wound sizes (SWS) on Day 0 were compared using ANOVA, and pairwise comparisons were performed using t-tests. Longitudinal analyses of relative wound size (RWS) values were performed using repeated measures ANOVAs with wounding time as a between-subjects variable. Group means for the day on which wounds were 50%, and 100% healed were compared using ANOVA and pairwise comparisons were performed using t-tests. Partial eta-squared was used to compare the magnitude of the effect of wounding time on healing rates. Linear regression analyses were used to validate photography in the L and D phases, to assess relations between CR amplitude measures and wound healing rates, and to assess relations between locomotor activity levels and wound healing. The level of statistical significance was set at α=0.05.

4.3 Results

Circadian responses to the DPS procedure: 26 hamsters yielded ENTR chronotypes, and 14 hamsters yielded ARR chronotypes and were included in subsequent procedures and analyses. Of the 8 hamsters that were not subjected to the 2 h light pulse on the night before the 3 h phase shift, all 8 yielded ENTR chronotypes and were also included in these experiments.

CR amplitude measures in Rounds 1 and 2: Relative to Round 1 ENTR hamsters, ENTR hamsters in Round 2 exhibited markedly lower amplitude CRs, as determined by mean (±SD): [1] Qp values at 24.0 h in the χ^2 periodogram (Round 1: 526±107; Round 2: 384±140; t_{24}=2.93, $p<0.01$), [2] PN values at 24.0 h in the LSP (Round 1: 121±44; Round 2: 78±59; t_{24}=2.13, $p<0.05$). ARR values for these CR metrics were substantially lower in ARR hamsters (χ^2: 151±31; LSP: 3.63±2.86) relative to ENTR hamsters ($p<0.0001$, ARR vs. Round 1 or Round 2 ENTR values, all comparisons). Thus, despite being categorically similar in circadian chronotype, the Round 1 and Round 2 populations of ENTR hamsters differed markedly in CR power. Consequently, in analyses of the effect of ZT on wound healing rates, data in ENTR hamsters were analyzed separately for these two rounds, as were data for ARR hamsters in Round 2.

Day 0 standardized wound sizes (SWSs): ANOVA indicated significant differences in Day 0 SWS across treatment groups ($F_{5,34}$=3.22, $p<0.05$). This effect was driven by: [1] smaller Day 0 wound sizes in Round 1 ENTR hamsters treated at ZT18 as compared to ARR hamsters treated at ZT03 (t_{14}=3.24, $p<0.01$) and ZT18 (t_{12}=2.84, $p<0.05$), and [2] smaller Day 0 wound sizes in Round 2 ENTR hamsters treated at ZT18 as compared to ARR hamsters treated at ZT03 (t_{11}=2.50, $p<0.05$). Pairwise differences were not evident in Day 0 SWS between any other treatment group ($p>0.05$, all comparisons).

Effects of time-of-day on wound healing: Among the robustly circadian ENTR hamsters in Round 1, there was a significant interaction between the time of skin wounding and the change in RWS over the next 17 days ($F_{17,238}$=2.89, $p<0.0005$; Fig. 4.1 A), characterized by a

pattern of slower healing in ZT18 relative to ZT03 wounds. Wound sizes of ZT18 hamsters tended to be greater than those of ZT03 hamsters on Day 2 ($p<0.07$), and were significantly greater than those of ZT03 hamsters on days 6-10 ($p<0.05$, all comparisons). Wounds of ZT03 hamsters achieved the 50% healing criterion >1 day sooner than those of ZT18 hamsters ($F_{1,14}=7.18$, $p<0.05$; Fig. 4.1 B); but 100% healing rate did not differ between ZT groups ($F_{1,14}=0.74$, $p>0.40$; Fig. 4.1 C).

Among the circadian ENTR hamsters in Round 2, there was a similar trend towards a significant interaction between wound timing and change in RWS ($F_{17,136}=1.65$, $p<0.06$; Fig. 4.2 A), again characterized by a pattern of ZT18 wounds healing slower than ZT03 wounds. RWS of ZT18 hamsters were significantly greater than those of ZT03 hamsters on day 3 ($p<0.05$), tended to be greater on day 4 ($p<0.07$), and were significantly larger on days 10-14 ($p<0.05$, all comparisons). Wounds of ZT03 hamsters also exhibited non-significant trends towards achieving the 50% and 100% healing criteria sooner than those of ZT18 hamsters ($F_{1,8}>3.52$, $p<0.10$; both comparisons; Figs. 4.2 B, 4.2 C).

Among ARR hamsters in Round 2, time-of-wounding did not affect the pattern of change in wound size over the next 17 days ($F_{17,204}=0.92$, $p>0.50$; Fig. 4.3 A). Wounds of ZT18 ARR hamsters did not differ from those of ZT03 hamsters on any day post-wounding ($p>0.05$, all comparisons). Wounding time affected neither 50% ($F_{1,12}=0.15$, $p>0.70$) nor 100% ($F_{1,12}=0.36$, $p>0.50$) healing rates among ARR hamsters (Fig. 4.3 B, 4.3 C).

Magnitude of treatment effects: We used partial eta-squared to compare the magnitude of the treatment effect (of wounding ZT) across the three comparisons described above (robustly-ENTR hamsters in Round 1, ENTR hamsters in Round 2, and ARR hamsters in Round 2). Partial

eta squared was calculated as the quotient of: ($SS_{between}$ / ($SS_{between}$ + SS_{error})) from the repeated measures ANOVA table for each analysis (Levine & Hullett, 2002). Partial eta squared values were 0.171, 0.171, and 0.071, for Round 1 ENTR, Round 2 ENTR, and Round 2 ARR hamsters, respectively.

Effects of chronotype on wound healing: In order to consider the influence of circadian power in the ENTR hamsters, wound healing was compared between Round 1 and Round 2 ENTR hamsters. ENTR hamsters wounded at ZT03 show a significant effect of Round ($F_{17,187}$=4.38, p<0.0001; Fig 4.4 A) in wound size, such that Round 1 hamsters had significantly smaller wounds on Days 7-11 (p<0.05), and trended toward smaller wounds on Days 12 and 13 (p<0.06). ENTR hamsters wounded at ZT18 show no significant difference between round (p>0.20; Fig 4.4 B). Round 1 ENTR hamsters trended toward achieving 50% healing faster than Round 2 hamsters for wounds at ZT03 (p<0.08; Fig 4.4 C), an effect which was significant for ENTR females wounded at ZT18 ($F_{1,11}$=3.89, p<0.03; Fig 4.4 D). There was no effect of round or wound ZT on 100% healing rate. Because there was a significant effect of round in the ENTR hamsters, these two ENTR populations were not collapsed in the following analyses with ARR hamsters.

Between Round 1 ENTR and ARR animals, there was a significant effect of chronotype at both wound times (ZT03: $F_{17,238}$=5.57, p<0.0001; ZT18: $F_{17,238}$=2.06, p<0.02, Fig. 4.5 A, D), characterized by ARR hamsters healing slower than Round 1 ENTR hamsters. ZT03 wounded ARR hamsters had larger wounds than Round 1 ENTR hamsters on Days 7-14 (p<0.04). ZT18 wounded ARR hamsters had larger wounds on Days 10-14 (p<0.002). In hamsters wounded at ZT03, ARR chronotype achieved 50% and 100% healed slower than Round 1 ENTR (50%:

$F_{1,14}$=10.59, p<0.007, Fig 4.5 B; 100%: $F_{1,14}$=8.57, p<0.02, Fig 4.5 C). Hamsters wounded at ZT18 trended toward faster healing in Round 1 ENTR for both 50% and 100% healing (p<0.07; Fig 4.5 E, F).

Between Round 2 ENTR and ARR hamsters, there was no significant effect of chronotype on wound size over the course of healing for either ZT03 (Fig 4.6 A) or ZT18 wounds (Fig 4.6 B). In Round 2, chronotype did not affect healing to 50%, but did influence healing to 100% in animals wounded at ZT03. ENTR hamsters healed faster than ARR hamsters ($F_{1,1}$= 5.37, p<0.05; Fig 4.6 C). This effect was not evident in animals wounded at ZT18 (p>0.05; Fig 4.6 D).

Relations between locomotor activity and healing rates: We next examined whether wound healing rate was associated with diurnal differences in locomotor activity (recorded prior to wounding) typical of that which would occur during the 8 h interval after wounding was performed. Across experimental rounds and ZTs, there were predictably significant between-group differences in activity during these 8 h epochs ($F_{5,34}$=12.7, p<0.0001; Fig. 4.7 A). In Round 1, among ENTR hamsters, mean activity was markedly lower between ZT03 and ZT11 (in ZT03-wounded hamsters) as compared to activity between ZT18 and ZT02 (in ZT18-wounded hamsters; t_{14}=5.84, p<0.0001); similar relations were obtained among ENTR hamsters in Round 2 (t_8=3.47, p<0.01), although the magnitude of the difference in activity was quantitatively smaller. Among ARR hamsters, mean activity between ZT03 and ZT11 (in ZT03 hamsters) did not differ from activity between ZT18 and ZT02 (in ZT18 hamsters; t_{12}=0.57, p>0.50; Fig. 4.7 A).

Separate linear regressions were performed for Round 1-ENTR, Round 2-ENTR, and Round 2-ARR hamsters (collapsed across wounding ZT), to assess relations between locomotor activity and wound healing. Among Round 1 ENTR hamsters, activity typical of that which would occur during the 8 h interval after wounding, was a significant positive predictor of the interval of time required to achieve 50% wound healing (R^2=0.37, df=15, $p<0.05$; Fig. 4.7 B). A categorically similar relation was obtained among ENTR hamsters in Round 2 (R^2=0.41, df=9, $p<0.05$; Fig. 4.7 C). Among ARR hamsters, however, no relation between these variables was observed ($R^2<0.01$, df=13, $p>0.80$; Fig. 4.7 D).

Identical regression analyses of activity on healing were performed using the 100% healing criterion as the dependent variable, but no significant relations were evident ($R^2<0.07$, df=9-15, $p>0.30$, all comparisons; data not illustrated).

Relations between CR power and healing rates: Lastly, to take advantage of the broad range of CR power across all animals in these experiments, an exploratory analysis was performed in which data from all rounds were subjected to a regression analysis, with circadian power (Qp values from the χ^2 periodogram) as the predictor variable and 50% healing rate as the dependent variable. Circadian power was a significant negative predictor of 50% wound healing values (R^2=0.20, df=39, $p<0.005$; Fig. 4.8 A). To assess whether these effects were being driven solely by the relatively lower Qp values in ARR hamsters, supplementary regression analyses were performed separately on ARR and ENTR data. Qp significantly positively predicted 50% wound healing rate among ENTR hamsters (R^2=0.16, df=25, $p<0.05$; Fig. 4.8 B). No significant relation was evident between Qp values and 50% healing rate among ARR hamsters ($R^2<0.01$, df=13, $p>0.90$; Fig. 4.8 C).

4.4 Discussion

The Disruptive Phase Shift (DPS) model successfully induced arrhythmia in Siberian hamsters. Analysis of locomotor activity designated animals as either circadian ENTR or ARR, meeting the criteria outlined above. ENTR hamsters could be ranked by robustness of entrainment, based on the amplitude of the χ^2 Periodogram. Using these chronotypic categorizations, results of these experiments clearly showed that rate of wound healing was influenced by time of injury, circadian integrity and circadian chronotype in female hamsters. ENTR hamsters were slower to recover from a wound in the early active period (ZT18) as compared to the early inactive period (ZT03), whereas ARR hamsters exhibited no circadian modulation of healing. ARR hamsters healed slower than ZT03 wounded ENTR hamsters, indicating that a functional circadian system is indeed essential for optimal recovery following cutaneous wound. Among ENTR hamsters, robustness of entrainment, as defined by CR power and locomotor activity, was a strong predictor of the circadian differences in wound healing.

Measurement techniques of wound area were successfully validated using separate wounds in both light and dark conditions. While the focal length of the digital camera did change under dim red light, compared to white light, the analyses used to calculate wound size compared to the standard did not change between lighting conditions. Thus, we describe and validate here that it is possible to obtain high resolution images of animals under two lighting conditions, and compare wound size using the same parameters of analysis.

In both groups of ENTR animals, wound size directly following skin punch was smaller in ZT18 compared to ARR females wounded at either time (vs. Round 1 ENTR), or ZT03 (vs. Round 2 ENTR). No differences in initial wound size were observed in ENTR animals wounded

at ZT03 compared to other groups. Experimenters were blind to circadian chronotype at the time of wounding, and photographs of wounds were completed within 30 seconds of skin punch, before animals could recover from anesthesia. Such a rapid change in wound size for the ZT03-ENTR hamsters implies that there is evidently an effect of circadian time in the immediate skin response to trauma, which is likewise dependent on a functional circadian system, as this ZT03 effect was not observed in ARR hamsters.

The reason for this ZT03 effect on initial wound size is not immediately clear. There is evidence supporting a variation in the capacity for immediate skin contraction based on time of day. Reflexive blood flow in the skin involves active vasoconstriction and vasodialation. The sensitivity of both of these systems has been shown to operate with circadian variation in humans (Aoki et al., 2001). Hemostasis is the first step in wound healing, restricting blood flow to the site of injury, and forming a clot in the wounded tissue (Diegelmann and Evans, 2004; Velnar et al., 2009). Thrombocytes mediate this response, and these cells exhibit clear circadian rhythms in aggregation and adhesion to leukocytes and endothelial cells, with activity peaking in the early active phase (Pritchett and Reddy, 2015; Scheer et al., 2011), the time at which we observed smaller initial wounds in ENTR hamsters. In Guinea pigs, mitosis around the edge of a wound in the tympanic membrane exhibits circadian variation (Reeve, 1977). Thus, it may be the case that a combination of hemostatic processes, and early cell proliferation at the site of injury resulted in immediate contraction of the wound site, more prominent at specific phases of the circadian cycle. This effect is most apparent in tissue damaged in the early active phase, in those animals with an intact, robust circadian rhythm. Such an inference is all the more supported by the absence of immediate wound size difference in the ARR hamsters.

The smaller initial wound size in ZT18-ENTR hamsters does not complicate the interpretation of the present data. Despite an initially smaller wound size, ZT18-ENTR animals had a larger inflammatory response, and slower subsequent wound healing. This effect was significant in the robustly entrained hamsters, and emerged as a statistical trend in the Round 2 ENTR animals. However, the effect size (as measured by partial eta squared) was comparable for both groups, indicating that the circadian variation was present in all ENTR hamsters. Thus, the rapid contraction of skin in the ZT18 hamsters does not seem to convey any defined advantage to the healing process, either in reduction of inflammation or in tissue repair and regeneration.

The phases of wound healing are defined by the recruitment of particular cell types to the site of injury, and the coordination of those cell types to remove foreign objects and bacteria and begin the process of regenerating and remodeling tissue (Velnar et al., 2009). In the early stages of inflammation, neutrophils act via phagocytosis and secretion of antimicrobial molecules to clear bacteria from the site of the wound. Next, macrophages and lymphocytes continue the process of phagocytosis, while also forming a structural connective matrix through which fibroblasts, collagen, endothelial and epithelial cells continue the process of repairing tissue in the proliferative phase of healing (Diegelmann and Evans, 2004). The magnitude of neutrophil trafficking to the site of tissue damage is defined by a circadian rhythm. In lung tissue of LPS challenged mice, recruitment of neutrophils, macrophages, and proinflammatory cytokines all show circadian variation dependent on the time of challenge (Gibbs et al., 2014). An endogenous circadian clock in several subtypes of macrophages determined magnitude of response to immune challenge, and that this response was present independent of circadian cortisol signaling (Keller et al., 2009). Coordination of the immune response following tissue damage is essential to the continued process of healing. The proliferative phase of tissue regeneration and repair

cannot begin until the wound has been cleared of harmful bacteria, and the cellular matrix has been formed for repair (Velnar et al., 2009). Timing of tissue damage then becomes an essential predictor for the speed of recovery if coordination of the cellular recruitment process to the site of the wound varies based on endogenous clocks in immune cells. Hamsters with robust circadian amplitude expressed a temporal difference in inflammation and subsequent tissue repair, with animals wounded during the early active phase taking longer to recover.

ARR animals did not exhibit any circadian differences in healing rate, and were slower to heal wounds, underscoring the likely importance of circadian coordination of events for optimal wound healing. Regardless of time of day, ARR animals exhibited the same magnitude of inflammatory response, and time to 50% and 100% healing. ARR hamsters lack a detectable circadian rhythm in multiple aspects of physiology and behavior (Grone et al., 2011; Prendergast et al., 2015; Ruby et al., 1996; Ruby et al., 2004), including blood leukocyte trafficking (Prendergast et al., 2013). The precise coordination of cellular signaling and response required for wound healing is also subject to circadian function, which is absent in the ARR chronotype. Circadian time of wounding did not affect ARR animals, but this chronotype did have delayed healing relative to ENTR hamsters. Round 1 ENTR hamsters healed significantly faster than ARR hamsters, and ARR hamsters had larger wounds over the later duration of healing. Round 2 ENTR hamsters exhibited the same general pattern compared to ARR hamsters, but the effect was non-significant. More rapid healing in ENTR hamsters compared to the ARR chronotype suggests a marked advantage in healing for hamsters with a functional circadian system. The presence of circadian rhythms contributes to the coordination of cellular recruitment to the site of the wound over the duration of healing. Absent these rhythms, the proliferative and reconstructive stages of wound healing and tissue repair required more time in the ARR

chronotype. Slower healing and larger wounds in the ARR chronotype leave the organism significantly more vulnerable to infection (Rojas et al., 2002).

The clearest effect in ENTR hamsters was the faster healing in ENTR hamsters wounded at ZT03. Indeed, ENTR hamsters wounded at ZT18 exhibited healing rates similar to those of ARR hamsters, suggesting that even in a circadian-intact animal, the time of injury can be just as detrimental to subsequent healing as complete loss of circadian coordination. It is therefore worth considering the mechanism(s) by which time of day influences the immediate and prolonged healing of an acute wound.

To accomplish this, we examined locomotor activity during the interval immediately following wound healing. In the photocycle used in the current experiment, hamsters typically engage in high levels of locomotor activity for ~6 h following ZT18, whereas ZT03 hamsters are in their rest phase for the next ~13 hours. Strikingly, in ENTR hamsters, locomotor activity strongly predicted the rate of healing. Greater amounts of locomotor activity in the 8 h window following wounding predicted slower healing to 50% in both populations of ENTR hamsters, but the same relation was not present in ARR animals. Immobilization of a wound markedly increases healing rate (Johnson, Ratner and Nelson, 1992). ENTR hamsters wounded at ZT18 are likely to groom, move the skin, and irritate a cutaneous wound, perhaps leading to an exacerbation of the inflammatory stage and a delay in healing rates. In contrast, ZT03 wounded ENTR hamsters are entering the inactive phase, and may leave the wound relatively immobilized. Such an effect of locomotor activity would also explain the absence of circadian modulation of wound healing in ARR hamsters: ARR hamsters are equally active in the light and dark phases. Thus, the effect of arrhythmia on wound healing may be mediated in part by a mechanism in which the circadian clock (or lack thereof) drives CRs in activity, which in turn

exacerbate, or fails to exacerbate, the initial behavioral response to a wound. By Day 1 post-wounding, each hamster would have completed a full active and inactive phase. Thus it is likely the immediate interaction between clock-driven activity and cutaneous wound repair that results in a larger inflammatory response and slower healing up to two weeks past initial injury.

Activity alone, however, is unlikely to fully explain the effects of time-of-day on wound healing. ARR animals lacked consistent and consolidated activity immediately following wounding, (which is seen in ENTR hamsters wounded at ZT18); indeed they are active around the clock, albeit at lower levels. Yet, ARR hamsters healed at the same rate or slower than the ENTR population. Activity immediately following wounding is predictive of healing rate in ENTR animals, but not sufficient to explain the differences in healing rates between chronotypes. It should be noted in the interpretation of these data that activity analyses were performed on all animals prior to wounding. Activity and grooming behavior may not be consistent in the 8h following the actual punch biopsy (wounding) itself, as compared to the same time period on a day when the animals were not disturbed.

Within the ENTR chronotype, power of the circadian waveform appears to play a large role in determining healing rate, regardless of circadian time of wounding. CR power as a measurement is a product of activity, and the strength of its coordination to the light-dark cycle. Round 1 ENTR hamsters had robust χ^2 (526±107) values, and demonstrated a clear difference in healing rate and inflammatory response in ENTR females, dependent on the time of wounding. Round 2 tended to replicate the results of Round 1, with a trend toward faster healing at ZT03. It should be noted, however, that ENTR animals included in Round 2 had a lower circadian power in activity analysis ($\chi^{2=}$ 384±140), potentially indicating a weaker coordination in circadian immunological response compared to Round 1 ENTR females. Compared to robustly ENTR

hamsters, ARR hamsters, with the lowest χ^2 values (121±44), had larger wounds over the duration of the inflammatory response, and tissue reconstruction. In both groups of ENTR hamsters, higher CR power predicted faster healing, strongly suggesting that robustness of the circadian rhythm conveys immunological advantages, potentially through coordination of the healing process.

Circadian integrity was remarkably important to the organismal-level immune response required for cutaneous wound healing. Circadian time of wounding predicted the speed of healing and the magnitude of the inflammatory response. ENTR animals had reduced healing rates in the evening compared to morning, with greater robustness of the rhythm correlated with faster healing. ARR hamsters lacked variation in time of day responses to wounding, but exhibited overall larger wounds for the duration of healing, and slower overall rate of healing compared to ENTR hamsters with robust CR power. Together, these data emphasize the necessity of an intact circadian system for normal wound healing, and considerable circadian variation among individuals with a functional circadian system. Such an outcome has enormous practical implications. Injuries sustained following extensive shift-work or transmeridian travel (which compromise circadian integrity) may heal at a reduced rate, increasing risk of infection, or excess disability (e.g. bed sores and limited mobility). The mechanisms by which the circadian system coordinates and organizes immune response over the multiple stages of wound healing remain to be fully understood.

Chapter 5: Neuroinflammatory alterations on the hypothalamic circadian gating of reproductive function.

5.1 Introduction

Regulation of reproductive function is influenced not only by the circadian system in many mammals, but also the overall state of the organism. This includes nutritional status (Bronson, 1988; Bronson and Marsteller, 1985; Schneider and Wade, 1989) and states of chronic or acute illness (Avitsur et al., 1997; Yirmiya et al., 1995). In female mammals, the function of the HPG axis is an energetically expensive process. Non-optimal conditions of acute and chronic physiological stress results in the cessation of reproductive function, in both males and females (Rivier et al., 1986; Rivier and Rivest, 1991; Beery et al., 2007). One of the most apparent and extreme examples of environmental influence on reproductive physiology is the complete absence of mating and reproduction during the winter months in seasonally reproductive species. As reviewed in the general introduction, several species, including Siberian hamsters, are seasonally dimorphic. In short-day, winter-like conditions, the gonads of both sexes regress, and circulating reproductive hormones typically fall several fold below concentrations in long-day conditions, and mating behaviors cease (Hoffman, 1973; Moffatt-Blue et al., 2006). Female hamsters cease 4-day estrous cycles and ovulation, and successful fertilization becomes physiologically impossible (Schlatt et al., 1993). Seasonal changes in reproductive physiology are considered adaptive due to the unfavorable conditions for gestation and rearing of offspring in the winter months, compared to the more optimal, less costly conditions in the summer months. In response to both short-and long-term environmental stressors, the reproductive

system is especially adaptive to the temporary cessation of function under unfavorable conditions, and rapid recovery when the energetic landscape becomes more favorable.

The reproductive system also demonstrates a physiological and behavioral interaction with the innate immune response. Along with the characteristic suite of sickness behaviors, activation of the innate immune system results in the suppression of sex behavior in female, but not male, rodents (Avitsur et al., 1997; Yirmiya et al., 1995). The suppression of sex behavior in females is mediated, at least in part, by the pro-inflammatory cytokine, IL-1β. Central or peripheral injection of IL-1β results in a response similar to an injection of LPS (Yirmiya et al., 1995). Additionally, injection of IL-1 receptor antagonist (IL-1ra) rescues sex behavior, along with a reduction in all sickness behaviors (Avitsur et al., 1997).

While the behavioral response to innate immune activation has been well documented, the neural mechanism by which the pro-inflammatory cytokine response suppresses reproductive function is not well understood. Following peripheral immune challenge, macrophages produce large amounts of pro-inflammatory cytokines. These cytokines act locally to further recruit cells for the inflammatory response, and also act as a messenger to the central nervous system in order to generate the acute phase response, or sickness response (Janeway et al., 2005). Pro-inflammatory cytokines access the brain partially via humoral mechanisms: circumventricular organs, or active transport across the blood-brain-barrier (Maier, 2003). Lesions of the vagus nerve results in the suppression or absence of acute sickness behaviors following peripheral immune challenge, suggesting a neural route of communication between the periphery and CNS (Maier et al., 1998). In the brain, microglial cells act as central surveillance and modulators of the neuroinflammatory response, capable of producing pro-inflammatory cytokines, and recruiting additional immune activation within the CNS (Ousman and Kubes, 2012). Peripheral

immune challenge rapidly activates the neuroinflammatory response, resulting in the onset of a multitude of sickness behaviors, including the temporary suppression of reproductive function.

In addition to behavior, immune challenge suppresses multiple aspects of reproductive physiology. A peripheral injection of the IL-1β is sufficient to suppress the LH surge in female rodents (Rivier and Vale, 1989; Iwasa et al., 2008). LPS injection also suppresses LH-stimulated ovarian estradiol production in vitro (Taylor and Terranova, 1996). In the medial pre-optic area (mPOA), GnRH and subsequent LH secretion is suppressed following injection of LPS, or either of the proinflammatory cytokines IL-1β or TNFα (Herman and Tomaszewska-Zaremba, 2010; Wananobe and Hayakawa, 2003). Such a result indicates that suppression of HPG function is not isolated to peripheral mechanisms, but instead originates in the neural mechanisms underlying reproductive function.

RF-amide neuronal populations in the hypothalamus are a likely candidate for the interaction between the HPG axis and immune activation. As reviewed in the general introduction, RFRP-3 typically acts as an inhibitor of GnRH. Release of inhibition from RFRP-3 permits the LH surge, and absent the release of RFRP-3 inhibition, the LH surge and subsequent ovulation will not occur (Kriegsfeld et al., 2010; Ubuka et al., 2011). *Rfrp-3* mRNA expression is upregulated in the DMH of male rats under conditions of acute and chronic restraint stress, indicating that physiological stress influences the hypothalamic regulation of reproduction (Kirby et al., 2009). Multiple species also show variation in RFRP-3 cell population density, based on sex, and photoperiod in seasonally breeding species (Henningsen et al., 2015; Revel et al., 2008; Ubuka et al., 2011). *Rfrp-3* expression was increased in rat hypothalamus following a high dose, but not low dose, peripheral LPS injection (Iwasa et al., 2014). However, suppression of reproductive behavior and physiology is apparent at relatively low levels immune challenge

(Li et al., 2007), suggesting the role of RFRP-3 in immunological response may occur in a more precise manner, possibly dependent on stage of estrous cycle, or on reactivity of specific RFRP-3 populations. RFRP-3 producing populations in the DMH receive direct input from the circadian system for the appropriately timed removal of negative feedback to GnRH, in order to permit the LH surge and behavioral receptivity to mating (Gibson et al., 2008). In Siberian hamsters, RFRP-3-containing cell bodies are distributed throughout the medial region of the hypothalamus, including the anterior hypothalamus, premammillary nucleus, DMH and ventromedial hypothalamus (VMH; Revel et al., 2008; Ubuka et al., 2011). Projections from these cells bodies were identified in the POA, septal nucleus, amygdala and arcuate nucleus in this species (Ubuka et al., 2011). RFRP-3 has been associated with the regulation of reproductive physiology (Gibson et al., 2008; Kriegsfeld et al., 2010; Li et al., 2007; Ubuka et al., 2011), reproductive behaviors (Bentley et al., 2006; Ubuka et al., 2012; Ubuka et al., 2014), and feeding behaviors (Tachibana et al., 2005; Tachibana et al., 2008), however, the functional role for individual RFRP-3-containing cell body populations and projections have yet to be determined. Innate immune response may prevent the normal precision in timing of RFRP-3 inhibition and disinhibition of normal GnRH function, and these acute changes may also be dependent on alterations of differential reactivity of individual RFRP-3 neuronal populations following acute immune activation.

The following experiments tested the hypothesis that innate immune activation suppresses normal circadian-dependent reproductive behavior and physiology via the differential regulation of RFamide expressing neuronal populations in the anterior and posterior hypothalamus. The first experiment considered the transcriptional expression of RFamides in the hypothalamus in LPS injected freely-cycling female Siberian hamsters (Experiment 5.1). The

second experiment aimed to determine if sickness induced characteristics of RFamide expression are specific to the day of proestrus, using ovariectomized steroid-primed females as a model (Experiment 5.2). In both experiments, we also sought to identify whether any responsiveness of *rfrp-3* to neuroinflammation was specific to anatomical subsets of RFRP-3 cells, via coarse dissections that bisected the RFRP-3 population into rostral and caudal populations. Together, these data define the role of RFamide populations and circadian dependent inhibition in the reproductive response to acute illness.

5.2 Methods:

Experiment 5.1:

Animals: All animals for this experiment were <6 months of age, and maintained in a 15L:9D photoperiod from birth. Body weights were recorded 12, 6 and 0 weeks prior to injection to determine stable baseline. Body weight was taken again on the day of injection. Animals were injected with either saline (n=33) or LPS (n=40) at ZT06. Tissue collections followed 6 h (n=11 SAL; n=15 LPS), and 12 h (n=12 SAL; n=11 LPS) after injection.

LPS injections and measures: All LPS and control injections were administered at ZT06. LPS was administered via intraperitoneal (i.p.) injection at a dose of 0.625 mg/kg, dissolved in sterile saline with an injection volume of 2.5 µl/g based on body weights taken at the time of injection (LPS isolated from *Escherichia coli* strain 026:B6, Sigma, St. Louis, MO). Control animals received 100 µl of sterile saline via i.p. injection. Food weight was also recorded at the time of injection. Following LPS or saline injection, animals remained undisturbed in the home cage until time of tissue collection. Tissues were collected at 6 h, or 12 h post-injection (ZT12

and ZT18, respectively). Food weight and body weight were recorded for each animal at the time of collection. Total food intake was calculated as the difference in food weight at injection minus food weight at tissue collection time, divided by the number of hours between the two time points. Food intake was presented as grams of food eaten per hour. Change in body weight was calculated as a percent difference between injection and collection. At tissue collection, hamsters were deeply anesthetized via isoflurane anesthesia (3.0% in O2), and blood was collected (300-400 µl) from the retro-orbital sinus using a sterilized Pasteur pipette coated with sodium heparin, Blood was collected into a heparinized (15 µl) tube, vortexed and placed on wet ice. While still deeply anesthetized, animals were rapidly decapitated and brain tissue was collected. Methods for brain tissue collection are detailed below.

Tissue collections: Whole brains were removed, and the anterior and posterior hypothalamus was microdissected via methods described in Prendergast et al. (2013). Briefly, upon exposure and visualization of the ventral surface of the hypothalamus, a coronal incision was made at the level of optic tract disappearance from the external surface of the brain. Such an incision bisected RFRP-3 medial hypothalamic neural populations into anterior and posterior sections, which were immediately flash frozen on dry ice. Representative images from the Allen Brain Atlas illustrate localization of hypothalamic microdissection via sagittal and coronal views (Supplementary Figures 1 and 2, respectively). Additionally, a representative publication identifying the anatomical localizations of RFRP-expressing cell bodies in the Syrian hamster brain provide visualization of hypothalamic cell populations (Kriegsfeld et al., 2006; Supplementary Figure 3), Remaining whole brain was bisected sagittally, and placed in RNALater solution (3ml; ThermoFisher Scientific) for later microdissection of cortex and

hippocampus, which were then flash frozen on dry ice. Uterine tissue was extracted and weighted. All dissected tissues were stored at -80 °C until RNA extraction.

Leukocyte quantification: For all experiments, blood collected from the retro-orbital sinus was processed for leukocyte quantification. 25 µl heparinized blood was immediately lysed following collection in 3% acetic acid solution (475 µl; 1:20 dilution). Remaining whole blood was centrifuged (1200 x g, 25 min), and plasma was stored at -80 °C for later analysis. Leukocytes were quantified in lysed blood less than 2 h following extraction. This methodology does not allow for quantification of leukocyte sub-types, but does permit an omnibus measure of circulating leukocyte concentration and trafficking (Prendergast et al., 2013). Leukocytes were manually quantified in duplicate (20 µl each) on a hemacytometer at 400× magnification. Experimenter was blind to injection type for all steps of quantification.

RNA extraction, cDNA synthesis and Quantitative olymerase chain reaction (qPCR): *il-1β* (Interlekin-1 beta), *tnf-α* (tumor necrosis factor alpha), and *rfrp-3* (RFamide- Related Peptide-3) relative mRNA expression was measured in anterior and posterior hypothalamus, via qPCR. Total RNA was extracted using RNeasy Plus Universal Minikit (Qiagen), according to manufacturer instructions. Extracted RNA was suspended in 32 µl RNase-free water (ThermoFisher Scientific). RNA concentration and quality for each sample was assessed using spectrophotometer (NanoDrop Technologies, Wilmington, DE). Complementary DNA (cDNA) was made for each sample using SuperScript III First-Strand synthesis system for RT-PCR (Invitrogen). qPCRs were performed using a BIORAD CFX384 system. Cycles followed these steps: Initial denature at 95 °C for 30 secs, followed by 39 cycles of 95 °C for 10 secs and

annealing temperature specific to target mRNA (Table 1) for 30 secs, and finally extension for 30 secs at 72 °C. Specificity for each gene of interest was determined by visualizing PCR product in 2.5% agarose gel prior to qPCR. Melting curves from each qPCR reaction were also inspected to determine the quality and specificity. iQ Sybr Green Supermix (BIORAD) was used for quantification of mRNA expression. PCR miner calculated reaction efficiencies and cycle thresholds (Zhao and Fernald, 2005). Efficiency for all reactions was between 0.8 and 1.2. Any reaction above or below those values was excluded from analysis. The expression of each gene of interest relative to the control gene, *gapdh*, was calculated using $2^{-(\text{delta-deltaCt})}$.

Experiment 5.2:

Animals: All animals for this experiment were >6 months of age, and maintained in a 15L:9D photoperiod from birth. Body weight for each animal was recorded 12, 6, and 1 week prior to injection to determine stable body weight, and again at the time of injection. One week prior to injections, all animals underwent ovariectomy and were steroid-primed as described below. Following recovery from surgery, animals were injected with either saline (n=39) or LPS (n=40) at ZT06. Tissues were collected at 6 h (n=13 SAL; n=13 LPS), and 12 h (n=13 SAL; n=13 LPS) post injection. Subsequent processing of tissues was performed as in Experiment 5.1.

Ovariectomy and Estradiol Implant: All females were ovariectomized via bilateral dorsal incisions under Ketamine-Xylazine anesthesia (70/5 mg/kg) and implanted with a subcutaneous capsule (0.5 mm silastic tubing) containing β-estradiol (Sigma Chemical Co. St. Louis, MO), a standard procedure to induce a daily LH surge (Donham et al. 1987). All hamsters were administered standard post-operative buprenorphine analgesic (0.01 mg/kg, s.c.) 2×/day for 2

days after surgery and monitored for the duration of recovery. LPS injection and tissue collection began 7 days following OVx+E.

LPS injections and measures: Following recovery from surgery, LPS injections and measures were the same as in Experiment 5.1.

Tissue collections: All tissues were extracted as in Experiment 5.1, flash frozen and stored at -80 °C until RNA extraction.

Leukocyte quantification: Quanitification of blood leukocytes was the same as in Experiment 5.1.

RNA extraction, cDNA synthesis and quantitative PCR: All RNA extraction, cDNA generation, and qPCR analysis was the same as described in Experiment 5.1.

Enzyme-linked immunosorbent assay (ELISA): In order to assess LH concentration at the time of tissue collection, frozen blood plasma was assayed via ELISA. Assay was performed using 96-well microtiter plates. Plates were first filled with 50 µl of bovine LHβ monoclonal Ab in PB, and incubated overnight at 4 °C. Following incubation, excess serum was removed, and plates were washed using 200 µl of 10 mm PBS containing 0.1% Tween 20. Wells were then blocked with 200 µl of 10 mm PBS with 1% BSA and 0.1% Tween 20 for 1 h at room temperature. 50 µl of sample (diluted 1:10 in assay buffer) or standard concentrations of mLH were added in duplicate, and incubated for 2 h at room temperature. Plates were washed

following incubation, and 50 µl of Rabbit polyclonal LH antibody was added to each well (1:10000 dilution) and incubated at room temperature for 6 min. Following additional wash, 50 µl Polyclonal Goat Anti-Rabbit IgG conjugated to horseradish peroxidase was added to each well (1:2000 dilution) and incubated for 1 h at 37 °C. Plates were washed once again, and OPD citrate buffer (100 µl) was added to each well, and allowed to develop in the dark for 30 min. Addition of 50 µl of 3 M HCl per well was added to stop enzyme reaction. The plate was immediately read at 490 nm using a reference of 655 nm. Use of 1:10 sample dilution was determined via pilot studies, in which 1:10, 1:20, 1:40, and 1:80 dilutions in high and low LH control samples were assayed in order to optimize reaction. Assay sensitivity was <0.15 ng/ml. Intra-assay variability was <3.1% for each plate, and inter-assay variability was <4.4%.

Statistical Analyses

Data were analyzed using the program StatView 5.0 (SAS Institute, Cary, NC, USA). Effects of injection type and collection time on all behavioral measures, leukocyte concentration, LH concentration, and relative mRNA expression was analyzed via ANOVA (2[Injection Type] × 2[Collection time] independent variables). Pairwise comparisons were performed with Fisher's PLSD, only if a significant omnibus F-statistic was obtained. A priori planned comparisons were performed without correction for family-wise error. ANOVAs and pair-wise comparisons were considered significant when $p<0.05$.

5.3 Results

Experiment 5.1

Food intake and body weight: Food intake and change in body weight provided a measure of sickness behaviors in response to LPS injection. Percent change in body mass (g) trended toward and main effect of injection ($F_{1,45}$=3.8, p<0.06) and had a significant main effect of collection time ($F_{1,45}$=4.1, p<0.05; Fig 5.1 A), such that LPS-injected hamsters decreased body mass at both collection times, while saline-injected hamsters deceased body mass only at 12 h post-injection. Food intake for LPS-injected females was reduced compared to saline-injected females ($F_{1,43}$=19.1, p<0.0001; Fig 5.1 B), an effect which was evident at both collection times (p<0.05, both comparisons).

Blood leukocyte concentration: Blood leukocyte concentration provided an omnibus measure of immune function, and insight to alterations in normal circadian trafficking. There was a main effect of collection time ($F_{1,45}$=10.8, p<0.003) and interaction between injection type and collection time ($F_{1,45}$=9.1, p<0.005) in blood leukocyte concentration. This interaction was characterized by an increase in leukocyte concentration for saline-injected females collected 6 h post-injection (ZT12, active phase), compared to saline-animals at 12 h post injection (ZT18, inactive phase), and all LPS-injected animals (p<0.003, all comparisons; Fig 5.1 C).

Anterior hypothalamic pro-inflammatory cytokine expression: There was a significant effect of injection type ($F_{1,36}$=5.2, p<0.05) and a non-significant trend toward an interaction between injection and collection time ($F_{1,36}$= 4.1, p<0.06; Fig 5.2 A) in anterior hypothalamic, *il-1β* relative mRNA expression. LPS-injected animals had significantly higher *il-1β* mRNA

expression at 6 h post-injection compared to saline animals at the same time ($p<0.01$). *Tnf-α* expression did not change by either injection type, or collection time ($p>0.05$; Fig 5.2 B). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression ($p>0.05$).

Anterior hypothalamic rfrp-3 expression: There was no effect of injection type or collection time in *rfrp-3* relative mRNA expression ($p>0.05$; Fig 5.2 C). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression ($p>0.05$).

Posterior hypothalamic pro-inflammatory cytokine expression: Injection type had a main effect on *il-1β* relative mRNA expression in the posterior hypothalamus ($F_{1,36}=16.14$, $p<0.0004$; Fig 5.3 A). At 6 h post-injection, LPS-injected females had higher *il-1β* mRNA expression compared to all other groups ($p<0.04$, all comparisons). *Tnf-α* expression trended toward a main effect of collection time ($F_{1,36}= 4.1$, $p<0.06$; Fig 5.3 B). Similar to results in *il-1β*, this trend was characterized by increased mRNA expression in LPS-injected animals at 6 h post-injection compared to all other groups ($p<0.05$). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression ($p>0.05$).

Posterior hypothalamic rfrp-3 expression: There were significant differences in posterior hypothalamic *rfrp-3* relative mRNA expression based on injection type ($F_{1,36}=6.39$, $p<0.02$; Fig 5.3 C). Tissue collected 12 h post-injection from LPS-injected animals had significantly decreased *rfrp-3* expression compared to saline-injected females at 12 h ($p<0.04$). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression ($p>0.05$).

Experiment 5.2

Food intake and body weight: There was no effect of injection type or collection time on percent change in body weight ($p>0.05$; Fig 5.4 A). Food intake in per hour was decreased in LPS-injected animals compared to saline-injected animals ($F_{1,47}=4.5$, $p<0.04$; Fig 5.4 B). This effect was driven by decreased food intake by LPS-injected animals compared to saline-injected animals at 6 h post-injection ($p<0.05$).

Blood leukocyte concentration: Replicating results of Experiment 5.1, circulating blood leukocyte concentrations demonstrated an effect of collection time ($F_{1,48}=7.73$, $p<0.008$) and interaction between collection time and injection type ($F_{1,48}=12.24$, $p<0.002$; Fig 5.4 C). This interaction was characterized by a decrease in LPS-injected leukocytes compared to saline at 6 h post-injection ($p<0.01$), and an increase in LPS-injected leukocytes compared to saline animals at 12 h post injection ($p<0.03$)

Anterior hypothalamic pro-inflammatory cytokine expression: In the anterior hypothalamus, there was a main effect of injection in *il-1β* relative mRNA expression ($F_{1,44}=10.83$, $p<0.003$; Fig 5.5 A), such that LPS-injected hamsters had increased expression at 6 h post-injection compared to saline injected-hamsters ($p<0.02$, both comparisons). Injection-type had a similar influence on *tnf-α* mRNA expression ($F_{1,44}=5.03$, $p<0.04$; Fig 5.5 B). As with *il-1β*, *tnf-α* expression in LPS-injected hamsters was increased at 6 h post-injection compared to saline-injected females ($p<0.03$, both comparisons). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression ($p>0.05$).

Anterior hypothalamic rfrp-3 expression: Anterior hypothalamic *rfrp-3* had a main effect of collection time ($F_{1,44}$=4.94, p<0.04), but not injection type (p>0.30; Fig 5.5 C). The main effect was driven by a decrease in LPS-injected *rfrp-3* at 6 h post-injection, compared to *rfrp-3* in saline-injected animals at 12 h post-injection (p<0.05). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression (p>0.05).

Posterior hypothalamic pro-inflammatory cytokine expression: LPS injection significantly increased both *il-1β* mRNA expression ($F_{1,47}$=22.3, p<0.0001; Fig 5.6 A), and *tnf-α* mRNA expression ($F_{1,47}$=13.88, p<0.0006; Fig 5.6 B) in the posterior hypothalamus. For both pro-inflammatory cytokines, LPS-injected females had increased expression at both collection times compared to all saline-injected females (p<0.04, all comparisons). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression (p>0.05).

Posterior hypothalamic rfrp-3 expression: Posterior hypothalamic tissue showed no effect of injection type or collection time in relative *rfrp-3* mRNA expression (p>0.05; Fig 5.6 C). There was no effect of injection type or collection time on control gene (*gapdh)* mRNA expression (p>0.05).

Plasma LH concentrations: In the LH ELISA there was no main effect of injection. However, time of collection did reveal significant differences in blood LH concentration ($F_{1,48}$=41.1, p<0.0001; Fig 5.7). In both LPS- and saline-injected females, plasma LH

concentration was increased at 6 h post injection (ZT12) compared to 12 h post injection (ZT18; $P<0.0006$, all comparisons).

5.4 Discussion

In both free-cycling and ovariectomized, steroid-primed female Siberian hamsters, a peripheral injection of LPS reliably induced a sickness response evident in behavior, blood leukocyte trafficking, and the neuroinflammatory response. Interestingly, both free-cycling and steroid-primed populations showed a complex response in RFamide mRNA expression in the hypothalamus following LPS injection. In steroid-clamped females, the LH surge persisted, despite the presence of a robust sickness response.

The behavioral sickness response in LPS injected hamsters was most evident in food intake behavior, while body mass loss had not yet manifested in many animals at 12 h post-injection. All hamsters were injected with either saline or LPS at ZT06, in the first half of the inactive phase, when entrained nocturnal rodents limit food intake under normal conditions (For review: Mistlberger, 1994). In both experiments, there was a decrease in hourly food intake in the LPS injected females, compared to the saline-injected females. This suppression of food intake persisted into the dark phase (12 h post-injection) in the free-cycling animals, whereas steroid-primed animals showed recovery at the 12 h collection time.

As an omnibus, physiological measure, circulating leukocyte concentrations in both experiments had a similar pattern following LPS or saline injection. Daily changes in blood leukocyte concentration persisted with normal variation in saline injected animals. Leukocyte concentration was high in the blood during the inactive phase (6 h post-injection, or ZT12), and significantly lower during the active phase (12 h post-injection, or ZT18; Dahbhar et al., 1994;

Prendergast et al., 2013). In contrast, blood leukocyte concentrations in LPS injected females failed to continue demonstrating normal circadian variation. In both free-cycling and steroid-primed hamsters, blood leukocytes remained suppressed following LPS injection, persistent into the active phase. Interestingly, although the neuroinflammatory response in the hypothalamus subsided, the normal circadian variation in circulating leukocytes remained suppressed.

The central neuroinflammatory response, measured by mRNA expression of the proinflammatory cytokines *il-1β* and *tnf-α*, showed a robust initial response to LPS injection in both experiments at 6 h post-injection in both the anterior and posterior hypothalamus. This response was diminished by 12 h post-injection. Between the two cytokines, IL-1β exhibited most consistency in response to LPS injection, while TNF-α showed greater variability in upregulation. Both TNF-α and IL-1β act to induce the acute phase response following innate immune challenge. However, TNF-α has primary local action, at the site of infection, while IL-1β functions in more of an endocrine fashion, with greater range of circulation. Due to these differential effects, the central neuroinflammatory response to peripheral challenge is driven by a persistent IL-1β response (Murphy, 2011). In both experiments, mRNA expression was increased for both cytokines in hypothalamic tissue following LPS injection. *Il-1β* shows a consistent robust increase in expression 6 h following injection, where as *tnf-α* demonstrates a less consistent, but present, inflammatory response.

Through multiple mechanisms, the presence of a robust sickness response in both populations of hamsters was established and characterized following peripheral LPS injection. Thus, the question motivating these procedures aimed to determine the role of anterior and posterior hypothalamic RFRP-3 populations in the suppression of reproductive function following immune activation. The design of these experiments hypothesized that RFRP-3 could

suppress reproductive function either via an increase in RFRP-3 following LPS injection, or by maintaining baseline inhibitory function through the time of the LH surge, when healthy females show a decrease in RFRP-3, thereby permitting the LH surge at a specific circadian time on the day of proestrus. Because RFRP-3 cell bodies are distributed throughout the medial hypothalamus, project to a variety of neuronal substrates, and have been functionally implicated in a variety of behaviors, we also hypothesized that the neuroinflammatory response to LPS may vary based on population location. As a preliminary approach to this issue, we coarsely characterized responses of the anterior and posterior (rostral and caudal) populations of RFRP-3 neurons to local neuroinflammation. In free-cycling females, there were no differences in *rfrp-3* expression in the anterior hypothalamus in response to LPS treatment. *Rfrp-3* expression in the posterior hypothalamus did not differ by injection type at 6 h post-injection (ZT12), which would be the approximate time of the LH surge on the day of proestrus. At 12 h post-injection (ZT18), saline-injected hamsters had increased *rfrp-3*, while LPS injected hamsters maintained 6 h post-injection levels of expression. This outcome suggests a complex interaction between the neuroinflammatory response, and circadian timing of reproductive function, such that LPS suppresses a rise in *rfrp-3* at ZT18, specifically in posterior hypothalamic *rfrp-3* populations. Gibson et al. (2008) demonstrate robust changes in RFRP-3 cell counts over the day of proestrus, which are absent on diestrus, suggesting that this response may depend on stage of estrous cycle on the day of LPS injection.

In contrast to free-cycling hamsters, *rfrp-3* mRNA in the posterior hypothalamus showed no response to LPS injection compared to saline injection in steroid-primed females. Interestingly, it was the anterior hypothalamic tissue, which exhibited increased *rfrp-3* mRNA expression at 6 h post injection, compared to 12 h, for both LPS and saline-injected females.

Thus, *rfrp-3* expression in steroid-primed females did not respond to LPS injection in either section of the hypothalamus, but did exhibit circadian-dependent changes in expression, in contrast with that of free-cycling animals. In both free-cycling and steroid-primed females, the direction of *rfrp-3* expression change was inconsistent with initial hypotheses.

In steroid-primed females, increased anterior *rfrp-3* expression at 6 h post-injection compared to 12 h post-injection was coincident with increased (but not necessarily peak) LH concentration. Such an outcome is seemingly in opposition with the current understanding of *rfrp-3* function, and requires inquiry into differential roles of specific RFRP-3-containing populations in the hypothalamus. Coincident increase in *rfrp-3* expression and LH concentration may have been observed due to the circadian time of the 6 h post-injection tissue collection. This collection may have occurred *after* the LH surge in steroid-primed females, when RFRP-3 was acting to reinstate negative feedback and suppress further secretion of LH (Gibson et al., 2008). The present data are not conclusive for this hypothesis, but such an explanation would indicate that the anterior population of RFRP-3 cells was more robustly responsive in this function. Higher relative *rfrp-3* expression in the anterior hypothalamus, compared to posterior hypothalamus in steroid-primed females (an effect absent in free-cycling females) also supports the role of the anterior RFRP-3 population in proestrus regulation of reproduction. Increased *rfrp-3* mRNA expression only in the anterior hypothalamus of steroid-primed females potentially indicates an increased responsiveness to estradiol, specific to this population of cells. Previous work has indicated that RFRP-3-containing cells express ERα, and are responsive to estradiol treatment, but robustness of reaction in individual cell populations remains to be determined (Kriegsfeld et al., 2006).

In steroid-primed animals, lack of RFRP-3 response following LPS injection may be further considered with respect to circulating LH concentrations at the time of tissue collection. While multiple studies have shown that a low to moderate dose of LPS is sufficient to suppress the LH surge (Rivier and Vale, 1989; Taylor and Terranova, 1996), results of this experiment indicated that LPS-injected steroid-primed females maintained LH concentrations comparable to that of healthy (saline-injected) females. Despite a multitude of sickness behaviors, and robust neuroinflammatory response, LPS-injected females maintained an elevated LH concentration, which may have possibly contributed to a lack of upstream hypothalamic response in RFRP-3.

Thus, we must consider that, although hamsters were indeed expressing overt sickness behaviors following LPS treatment, and exhibiting marked increases in hypothalamic proinflammatory cytokine expression, reproductive function was unaffected (as inferred by the absence of an effect of LPS on LH). Although this suggests the use of a sub-threshold LPS stimulus—it can only be regarded as sub-threshold for the purposes of suppressing reproductive physiology (neuroinflammation and sickness behaviors, as well as leukocyte responses were robust) in hamsters. This level of neuroinflammation is sufficient to suppress LH surges mice and rat (Avitsur et al., 1997; Yirmiya et al., 1995). In this light, the data are remarkable and suggest a degree of resilience in the HPG axis of female hamsters that is not evident in other laboratory species.

The results of these experiments indicate that RFRP-3 is minimally responsive to high levels of neuroinflammation in Siberian hamsters, this opens the possibility that inflammation-induced changes in other neuronal mediators of reproduction may exhibit a robust response to LPS, and subsequent suppression of reproductive function. Kisspeptin-containing cells in the AVPV of the hypothalamus, as introduced in Chapter 3, are one such candidate. Kisspeptin may

be acting as a mediator to suppress positive feedback at the time of the LH surge. A high, but not moderate, dose of LPS does suppress *kiss1* mRNA expression in ovariectomized (not steroid-primed) rats (Iwasa et al., 2008; Iwasa et al., 2014). These data strongly implicate Kisspeptin as a mediator of reproductive suppression, but do not define the interaction with RFRP-3 function as it occurs under conditions of the proestrus sickness response.

Both RFRP-3 and Kisspeptin-containing cell populations receive direct input from the SCN, and function in a circadian-dependent manner, specific to the day of proestrus (Gibson et al., 2008; Robertson et al., 2009; Williams et al., 2011). While RFRP-3 was not robustly responsive to LPS, despite marked neuroinflammatory response, Kisspeptin remains a potential candidate for circadian-dependent suppression of reproduction following LPS injection. In this experimental design, it would be especially interesting to establish the Kisspeptin response to inflammation, in a model, which maintains LH concentrations to comparable to healthy females. If Kisspeptin is suppressed by neuroinflammatory response, but LH increases remain, it would suggest that GnRH or LH populations are maintaining function, independent of RF-amides, possibly via direct SCN-GnRH projections. The outcome of present experiments indicates that while neuroinflammation suppresses reproductive behavior, it likely does so through a complex background of alterations in positive and negative feedback, specific to phase of the estrous cycle, and dependent on time of injection and consequently acute circadian disruption.

Free-cycling and steroid-primed female hamsters had a robust sickness response in behavior and physiology following peripheral LPS injection. This procedure is sufficient to suppress the LH surge and reproductive behavior in previous literature, but results in steroid-primed hamsters indicated the persistence of LH elevation prior to the onset of the dark phase. In both experiments, *rfrp-3* mRNA expression in the hypothalamus did not demonstrate a response

consistent with the hypothesis that suppression of reproductive behavior is due to increased RFRP-3 inhibition. Rather, circadian-dependent changes in reproductive response do not seem to act via a negative feedback mechanism alone, but may also depend on an interaction with suppressed positive feedback, mediated by Kisspeptin. Future experiments will look at the contribution of Kisspeptin, in order to determine the role of positive feedback in the circadian mediated reproductive response following innate immune activation.

Chapter 6: General Discussion

The purpose of these experiments was to consider the circadian system and its influence on the function of reproduction and immune processes. Some experiments in this work used a non-invasive model of chronic circadian disruption (Chapters 3 and 4) in order to determine how the state of the circadian system influenced reproductive and immune processes; other experiments examined the interactions between these two systems (Chapter 5). Experiments in Chapter 3 determined, in contrast to much of the existing literature, that circadian arrhythmia disrupts the timing of the LH surge, but does not eliminate the surge itself- and does not inhibit reproduction and fertility in female Siberian hamsters. Using the same arrhythmia model, Chapter 4 established the importance of the circadian clock to normal innate immune function (wound healing). Healing was inhibited by behavioral circadian arrhythmia and was also impacted by time of day in animals that were circadian competent. Part of the time-of-day effect may be explained by locomotor activity effects on skin mobility—indirect effects of the circadian clock's timing of activity / rest cycles. Finally, Chapter 5 considered the influence of innate immune challenge to suppress another function under circadian influence: the reproductive system. Results of this experiment demonstrated that an innate immune challenge sufficient to elicit robust sickness response and a high level of neuroinflammation was inadequate to suppress the LH surge and did not upregulate hypothalamic *rfrp-3* gene expression. Together, these data define robust interactions between these three systems, and a previously unrecognized capacity for adaptation in order to maintain normal output under conditions of increased allostatic load.

The use of an alternative model of circadian arrhythmia in Siberian hamsters permits an insight into the role of circadian timing on reproductive function, which had not been previously

observed. In contrast with much of the existing literature, arrhythmic female hamsters exhibited much more modest deficits in fertility and reproductive function. While arrhythmia induced via constant bright light, SCN lesions, or clock-gene manipulations consistently results in robust reproductive compromise, the disruptive phase shift did not replicate these outcomes. Such a result emphasizes the necessity of comparative and convergent research. Indeed, each model of arrhythmia has its own distinct methodological advantages and disadvantages, including the relatively new DPS model in Siberian hamsters. A good amount of work has been accomplished to consider behavioral (Prendergast et al., 2014; Ruby et al., 1998; Ruby et al., 2004), clock-gene expression (Barakat et al., 2005; Grone et al., 2011), immunological (Prendergast et al., 2013), and cognitive (Fernandez et al., 2014; Ruby et al., 2008; Ruby et al., 2013) consequences of behavioral arrhythmia in this model. However, the precise characteristics and mechanisms underlying DPS arrhythmic hamsters remain to be determined. Nevertheless, when all methodologies of circadian disruption are applied and compared, it is possible to develop a more complete understanding of the mechanism by which these two systems interact, and the differential roles of the components involved.

Recent increased understanding for the role of Kisspeptin and RFRP-3 in positive and negative feedback mechanisms of the HPG permit greater insight into potential neuronal mechanisms by which behavioral arrhythmia may inhibit circadian-mediated function (e.g., the LH surge). In the present study we used a dose of LPS that was in some respects sub-optimal—LH surges persisted. On the other hand, hamsters injected with LPS exhibited profound sickness behaviors and neuroinflammation. It may be the case that these traits (LH vs. behavioral sickness symptoms) have different thresholds for cytokine inhibition. Alternatively, in light of the strong hypothalamic cytokine responses in LPS treated hamsters, it may be the case that hamsters are

resilient/refractory to even moderate to large LPS challenges. Absent an LPS-induced change in LH, however, insights on the role of RFRP-3 in neuroinflammation-induced LH suppression are not possible. However, this experiment does permit the conclusion that an immunological challenge sufficient to induce severe sickness and neuroinflammation does not inhibit LH. Thus these outputs and their underlying systems can be disentangled in this regard. It would be interesting in future research to determine if sex behavior is inhibited by a similar dose of LPS in female hamsters. Different traits evidently exhibit different thresholds for inhibition in response to inflammation. Analyses of Kisspeptin would be informative in this model as well. If Kisspeptin were to be inhibited by a neuroinflamatory challenge that fails to inhibit LH, such an outcome would raise doubts about the necessity of Kisspeptin in the generation of LH surges under conditions of experimental illness.

Future studies may also potentially benefit by further investigating the role of these the Kisspeptin and RFRP-3 neuronal populations under conditions of circadian arrhythmia. There is strong evidence for an oppositional role of Kisspeptin and RFRP-3 in the induction and suppression of the LH surge (Kinoshita et al. 2005; Smith et al. 2006; Robertson et al. 2009; Kriegsfeld et al., 2010; Xu et al. 2011). These data warrant consideration of the functional role of both neuronal populations in the ARR female, compared to function in the ENTR female. Such an experiment, accomplished using a comparative approach of arrhythmic models, would continue to define a precise mechanism by which reproductive function is changed based on the status of circadian input. One would hypothesize that in the ARR hamster exhibiting randomly timed LH surges, that Kisspeptin and or RFRP-3 are synchronized in time with the LH surge. Alternatively, arrhythmia may induce tonic disinhibition of LH, and direct circadian (SCN)

projections to the GnRH neurosecretory population may be driving the arrhythmic LH surges in ARR hamsters.

Abundant data point to negative functional consequences of chronic transient circadian disruption (Castanon-Cervantes et al., 2010; Davidson et al., 2006; Fonken et al., 2012; Gibson et al., 2010; Kott et al., 2012). In Chapter 4, wound healing was delayed in ARR females, but was also delayed in circadian ENTR females receiving a wound just prior to the active phase. While behavioral arrhythmia is not optimal for immune function, the consequences of immune challenge at the "wrong" time of day seem to be comparably detrimental. Thus, the outcome of these experiments suggest that a steady state of arrhythmia may be unfavorable for optimal immune health, but in some cases no more deleterious than immune challenge at select phases of the cycle. Potentially, the increased negative outcome from chronic jet-lag research is due to the persistent involuntary shifts in the functional clock. A comparison of wound healing outcome following chronic jet-lag or persistent arrhythmia would be informative on the issue of the severity of consequences in different models of disrupted entrainment.

Although the DPS arrhythmia model is a contrived laboratory model, plasticity in the circadian system is evident under natural conditions. It has been recognized for quite some time that the circadian clock is not completely functional, much less synchronized with the environment at birth in mammalian species (Dolatshed et al., 2010; Kittrell and Satinoff, 1986; Reppert et al., 1984), and in some arctic species during summer and winter (Lu et al., 2010; van Oort et al., 2005). What has received relatively less attention, until recently, are the complimentary changes in the maternal clock during gestation and lactation (Prendergast et al., 2012). Wang et al. (2014) also described a progressive degradation in CR amplitude and robustness over gestation and into lactation. Such a dramatic change in the circadian clock, and

the subsequent recovery of circadian rhythms after weaning of the offspring is a sterling example of chronotype plasticity that reflects adaptation to environmental conditions, and does not necessarily give rise to permanent dysfunction. Nutritional requirements of altricial mammalian offspring require that maternal investment not be limited to specific hours of the day or night. The capacity of the maternal clock to temporarily permit arrhythmia increases the chances for offspring survival. Flexibility in the circadian system reduces maternal allostatic load during this time, which would otherwise be encountered under conditions of behavioral entrainment. In this capacity, an already ARR female may actually have a behavioral advantage in offspring care over the circadian adjustments required of the ENTR female. A fundamentally interesting question, permitted by the results of these data, would be to consider immune function during gestation and lactation in an ARR and ENTR females. Such an experiment would explore the interaction of reproductive changes in immune function under conditions of a consistent arrhythmia (ARR), and transient arrhythmia (ENTR).

Persistence of comparably normal *rfrp-3* mRNA expression following LPS injection strongly supports a notion of redundancy in reproductive responses to neuroinflammation. Considering the resiliency of the reproductive system under conditions of permanent behavioral arrhythmia in this species, it becomes less surprising that Experiment 5.2 demonstrated persistently increased LH concentrations under conditions of a robust sickness response. One unexplored question, however, is how the circadian-mediated Kisspeptin neuropeptide system responds to neuroinflammation at different stages of the estrous cycle. While *kiss1* mRNA expression changes in moderate doses of LPS, it is only under high doses that alterations in both *rfrp*-3 and *kiss1* are observed in ovariectomized, non-steroid-primed rats (Iwasa et al., 2008; Iwasa et al., 2014). Suppression of sex behavior is observed following low doses of LPS or IL-

1β injections in multiple species (Avitsur et al., 1997; Herman and Tomaszewska-Zaremba, 2010; Rivier and Vale, 1989; Wananobe and Hayakawa, 2003; Yirmiya et al., 1995). However, rarely in these experiments is the time of injection reported, relative to the time of lights on or lights off. Such detail is essential for a mechanistic understanding of reproductive and immune response- both of which are dependent on time of day. Thus, while it would seem that both Kisspeptin and RFRP are involved in the suppression of reproductive function, inconsistency in immunological and reproductive dose-response is likely due to variability in circadian time of injection, and stage of estrous cycle.

Taken together, data from these experiments demonstrate novel interactions between the circadian, reproductive and immune systems. Circadian arrhythmia is sufficient to disrupt certain aspects of reproductive and immune function; immune activation alters circadian and reproductive responses; some aspects of the sickness response are mediated by reproductive condition. Perhaps of equal importance to our understanding bidirectional interaction is a greater appreciation for the resiliency of these systems in response to perturbations (arrhythmia, simulated illness). Entrainment of the circadian system permits synchronization of internal function to the external environment. While disruptive, changes to environmental orientation are not necessarily untenable to the organism. The outcomes of these experiments highlight the capacity for successful adaptation to change in a traditionally stable environment and the ability to preserve key physiological function in the face of extreme challenges.

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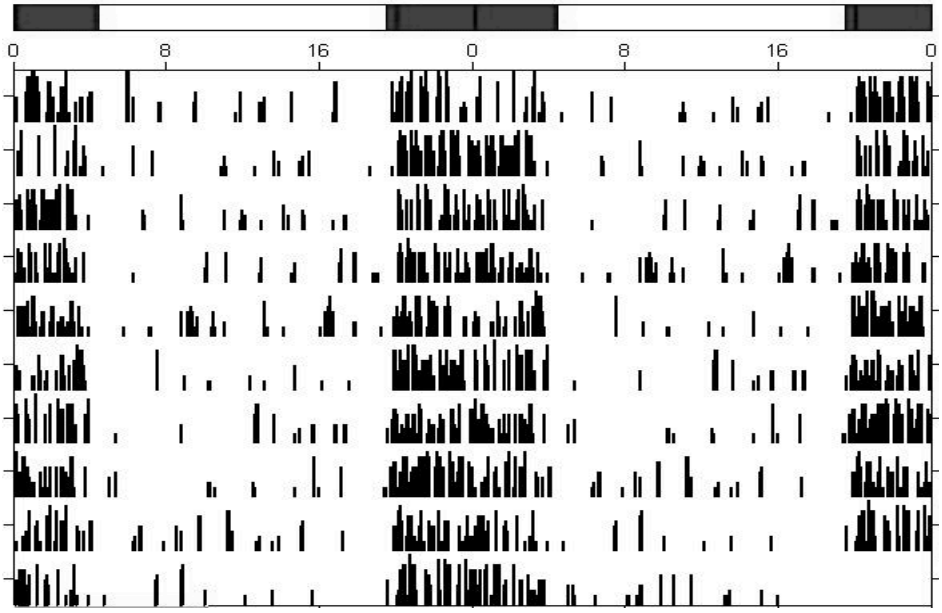
Appendix A: Tables

Table 1. Primers used in qPCR analysis

Gene	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size	Annealing Temp (°C)
gapdh	TTCTTGTGCAGTGCCAGCCTCG	CTGTGCCGTTGAACTTGCCGTG	207	60
rfrp-3	GTTGACTTTAGCCACTTCAAGC	GTGCCCTGGTACTTCTGTCC	287	61
tnf-α	CAACAAGGAGGAGAAGTTCCCA	GTGAGGAGCACGTAGTCGG	251	61
il-1β	GCACTACAGGCTCCGAGATG	CTTGGGATCCACACTCTCCAG	247	59

A. Entrained



B. Arrhythmic

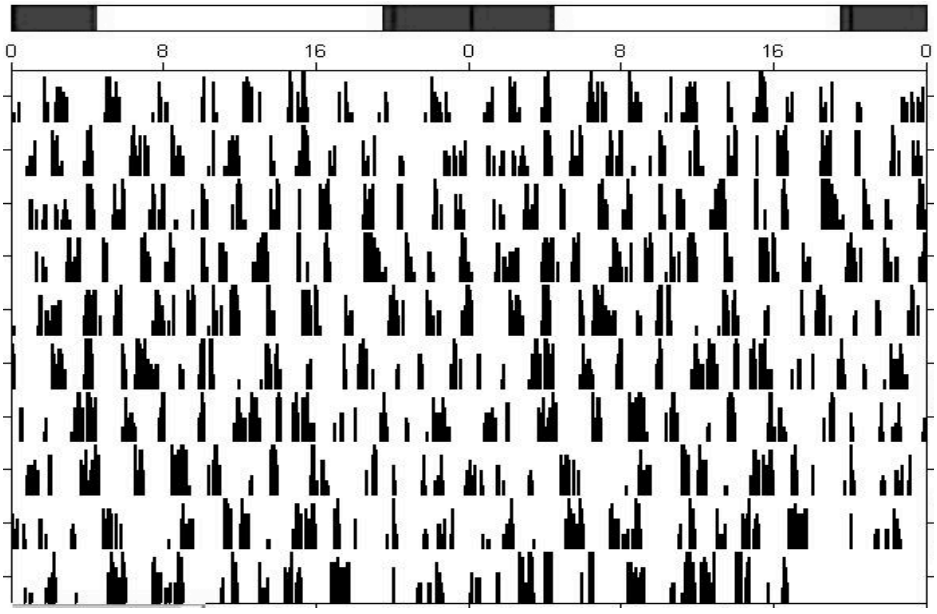


Figure 2.1. Locomotor activity of entrained and behaviorally arrhythmic female hamsters. Representative double-plotted actograms from (A) Entrained and (B) Arrhythmic female Siberian hamsters. Actograms are drawn to same scale (0-10 counts/bin). Clock time is indicated on the horizontal axis at the top of each actogram, along with light (white) and dark (black) phases of the 16L:8D photoperiod.

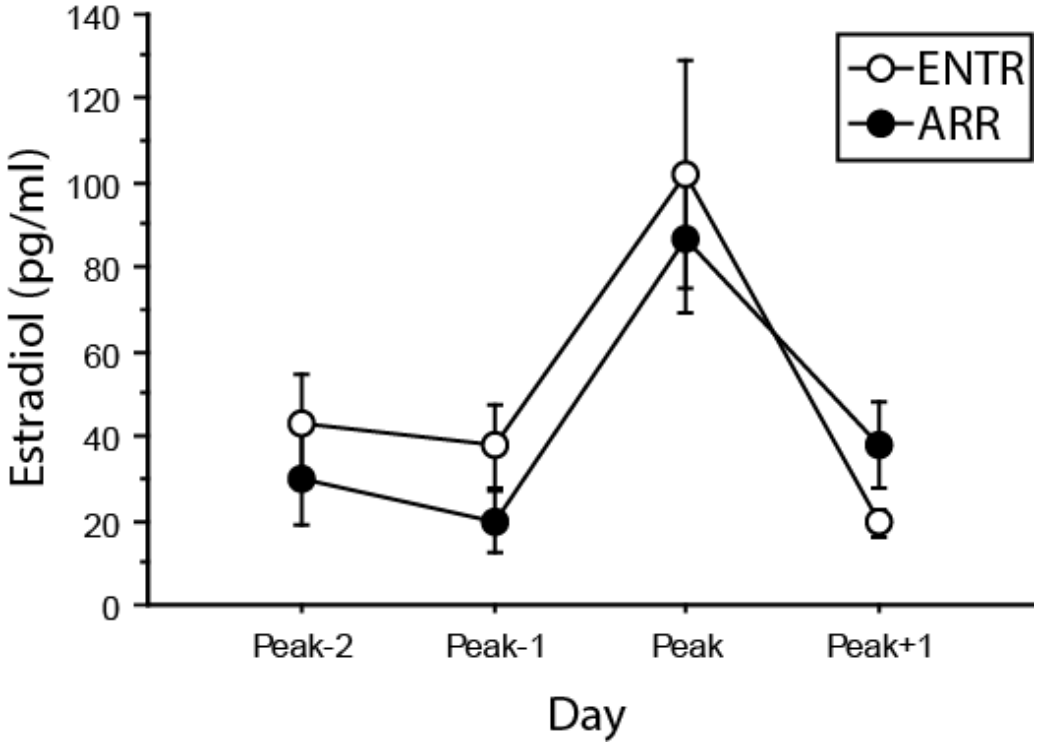


Figure 3.1. Concentrations of Plasma Estradiol on four consecutive days in ENTR and ARR females. Mean ± SEM blood estradiol (pg/ml) taken for four consecutive days at ZT10 for both ENTR (white circles; n=12) and ARR (black circles; n=12) female hamsters. Because day of proestrus is not able to be determined in this species, data are arranged around the day of highest estradiol. ARR and ENTR females do not differ in either pattern of estradiol over the 4-day estrus cycle, or the amplitude of estradiol on day of peak ($p>0.05$).

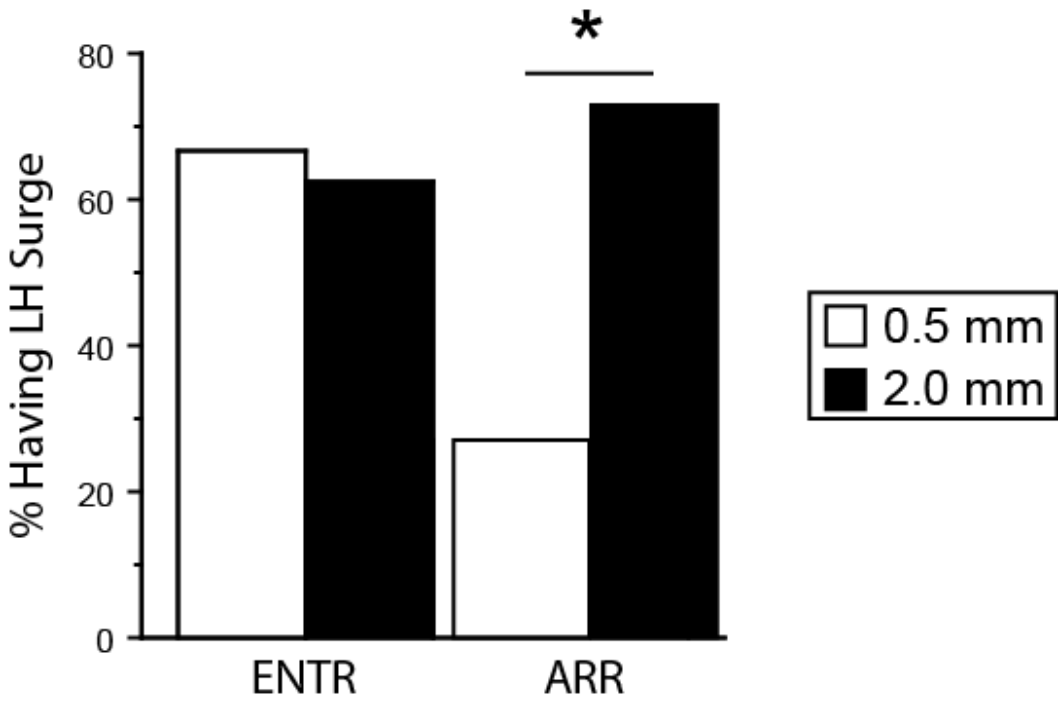


Figure 3.2. Effect of estradiol implant size on LH surge occurrence. Chi-square analysis of the proportion of ENTR (n=33) and ARR (n=29) females having an LH surge, following OVx+E with either a 0.5 mm or 2.0 mm estradiol implant. ARR females with a 0.5 mm estradiol implant had significantly fewer surges than ARR females with a 2 mm estradiol implant (*$p<0.05$). This effect of implant size was not replicated in ENTR females.

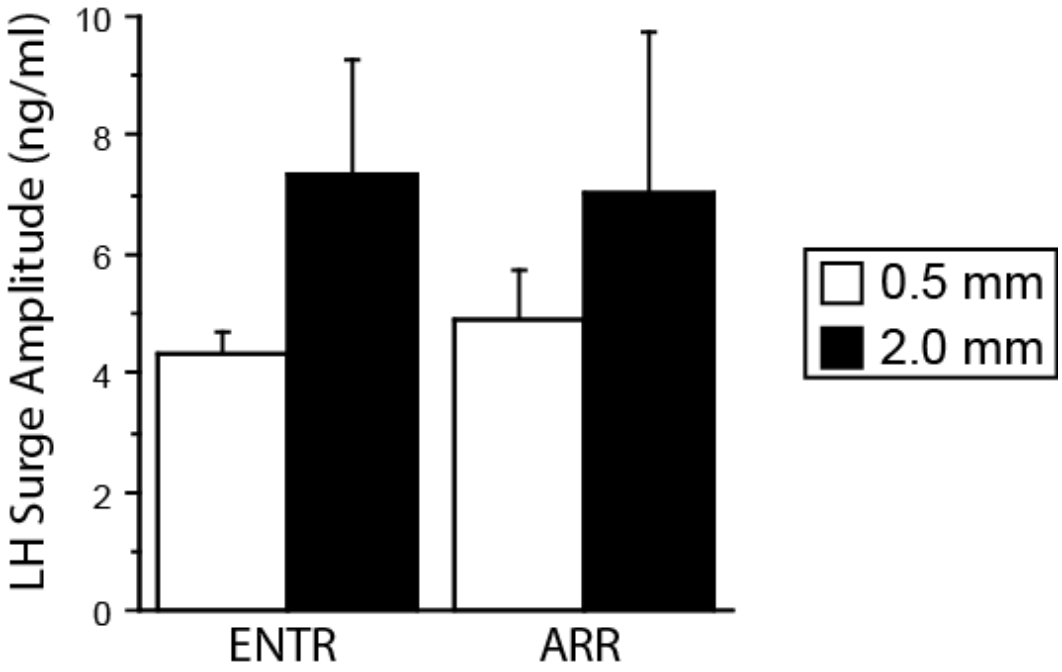


Figure 3.3. Amplitude of LH surge. Mean ± SEM measure of LH surge amplitude (ng/ml) for ENTR and ARR females with 0.5mm and 2.0mm estradiol implants. LH surge is defined as 4 standard deviations above the mean measure of blood LH during a non-surge time (ZT06) in ENTR females. There was no effect of behavioral phenotype or implant size on the amplitude of the LH surge ($p>0.05$).

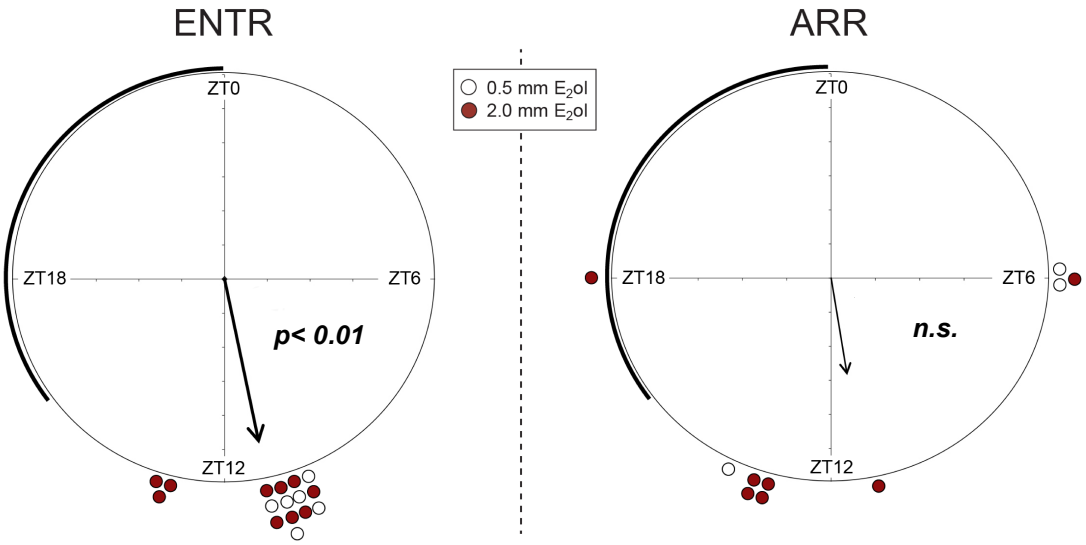


Figure 3.4. Timing of the LH surge in ENTR and ARR females. Mean ± SEM direction and concentration of LH surge time in a circular distribution (ZT0- ZT24). Time of LH surge and implant type is represented for individual females by colored dots on the circumference of the graph (2.0 mm implant= white; 0.5 mm implant =black). Arrow direction indicates mean time of LH surge, while length of arrow represents strength of association. (A) ENTR females (n=16) have a significant concentration of LH surge time ($P<0.001$). (B) ARR females (n=10) do not have significant concentration of LH surge time ($p>0.01$). A conservative alpha ($\alpha=0.01$) was used in this measure as samples were not taken at evenly distributed times over 24 hours.

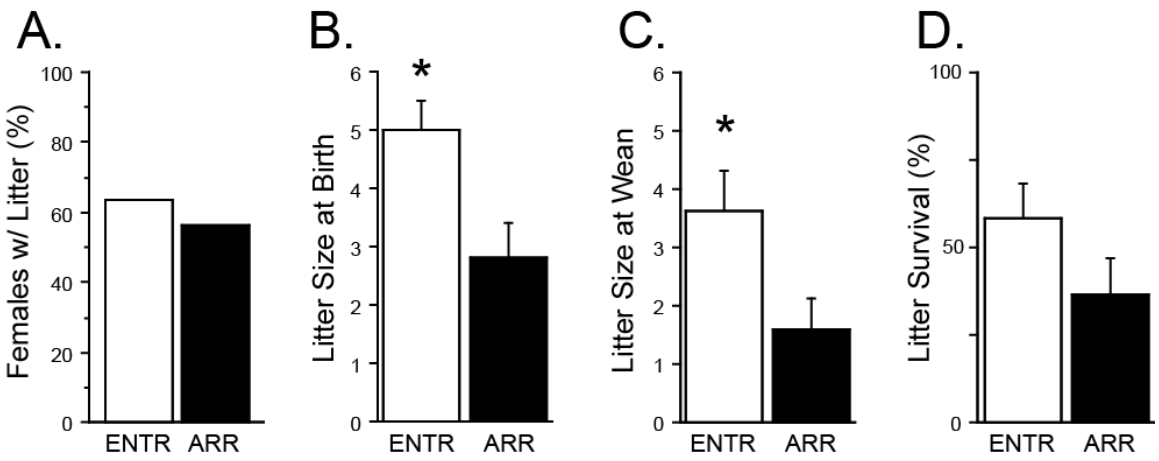


Figure 3.5. Reproduction and fertility performance following 5-day cohabitation. Chi-square analysis for (A) proportion of ENTR and ARR females delivering a litter following cohabitation. Mean ± SEM for (B) litter size at birth, (C) litter size at weaning, and (D) percent litter survival for ENTR (n=30) and ARR (n=32) dams. Litter size at birth and weaning was larger for ENTR compared to ARR dams (*$p<0.05$), but not for proportion for females pregnant or litter survival.

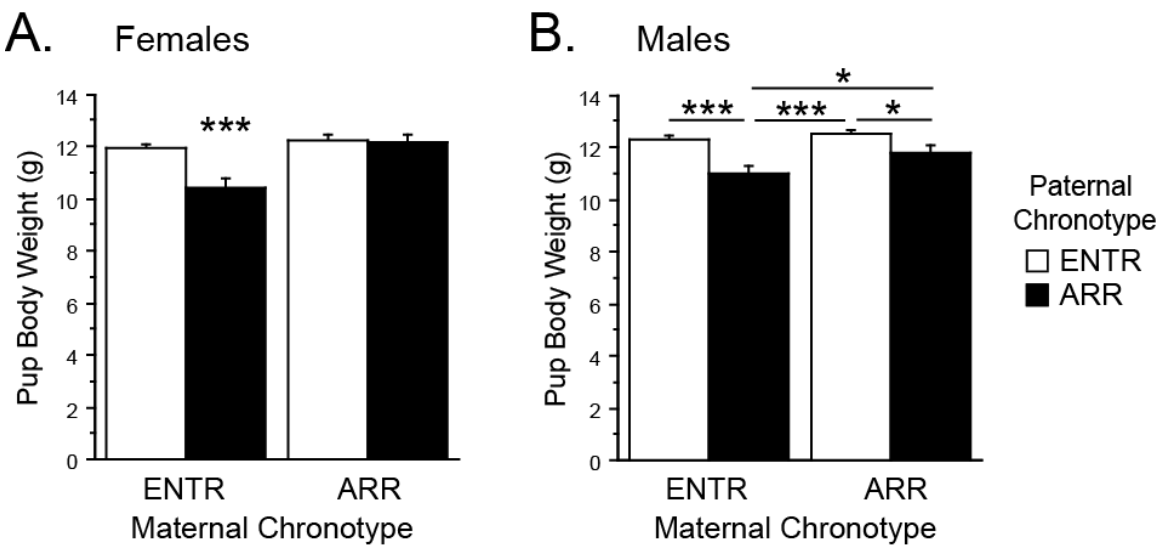


Figure 3.6. Body weight of individual pups at weaning in 90-day cohabitation paradigm. Mean ± SEM body weight (g) for (A) female and (B) male pups born to ENTR or ARR dams, and either a ENTR (white bars) or ARR (black bars) sire (Male: AA=70, AE=102, EA=124, EE=156; Female: AA=60, AE= 74, EA=86, EE=128). For all pups ARR sire resulted in lower body weight at weaning (*$p<0.05$, ***$p<0.0001$).

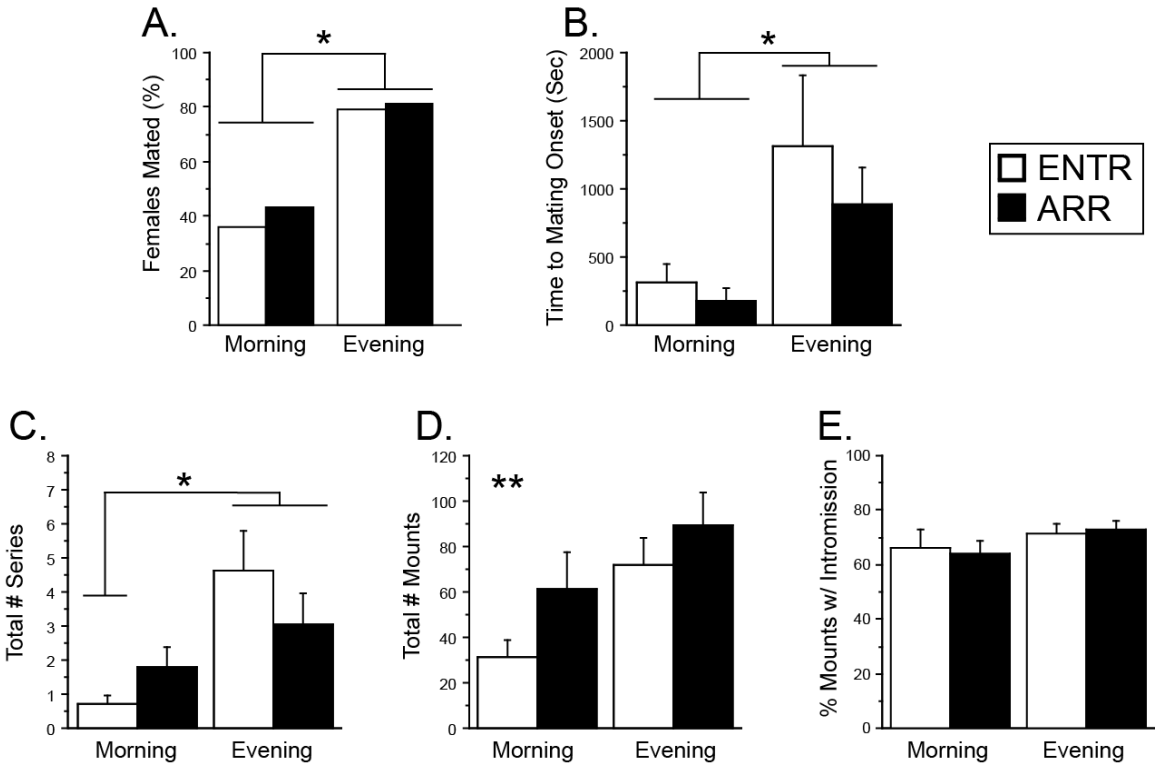


Figure 3.7. Reproductive behaviors in 3-h timed pairings. Chi-square analysis for (A) proportion females mating with an ENTR male during evening (ZT15-ZT18) or morning (ZT04-ZT07) pairings. Mean ± SEM for (B) latency to first series onset (in seconds), (C) total number of completed series, (D) total number of male mounts, and (E) number of intromissions as a proportion of total mounts was compared between ENTR and ARR females paired in the morning (ENTR=25, ARR=23) and evening (ENTR=24, ARR=16). Mating occurrence was lower in the morning compared to the evening (*$p<0.05$), but showed no phenotype differences. Latency to first series was lower in the morning (*$p<0.05$), had fewer completed series (*$p<0.05$), and total male mounts (**$p<0.01$ vs. all other groups). Females were equally likely to permit intromission regardless of time of day or phenotype.

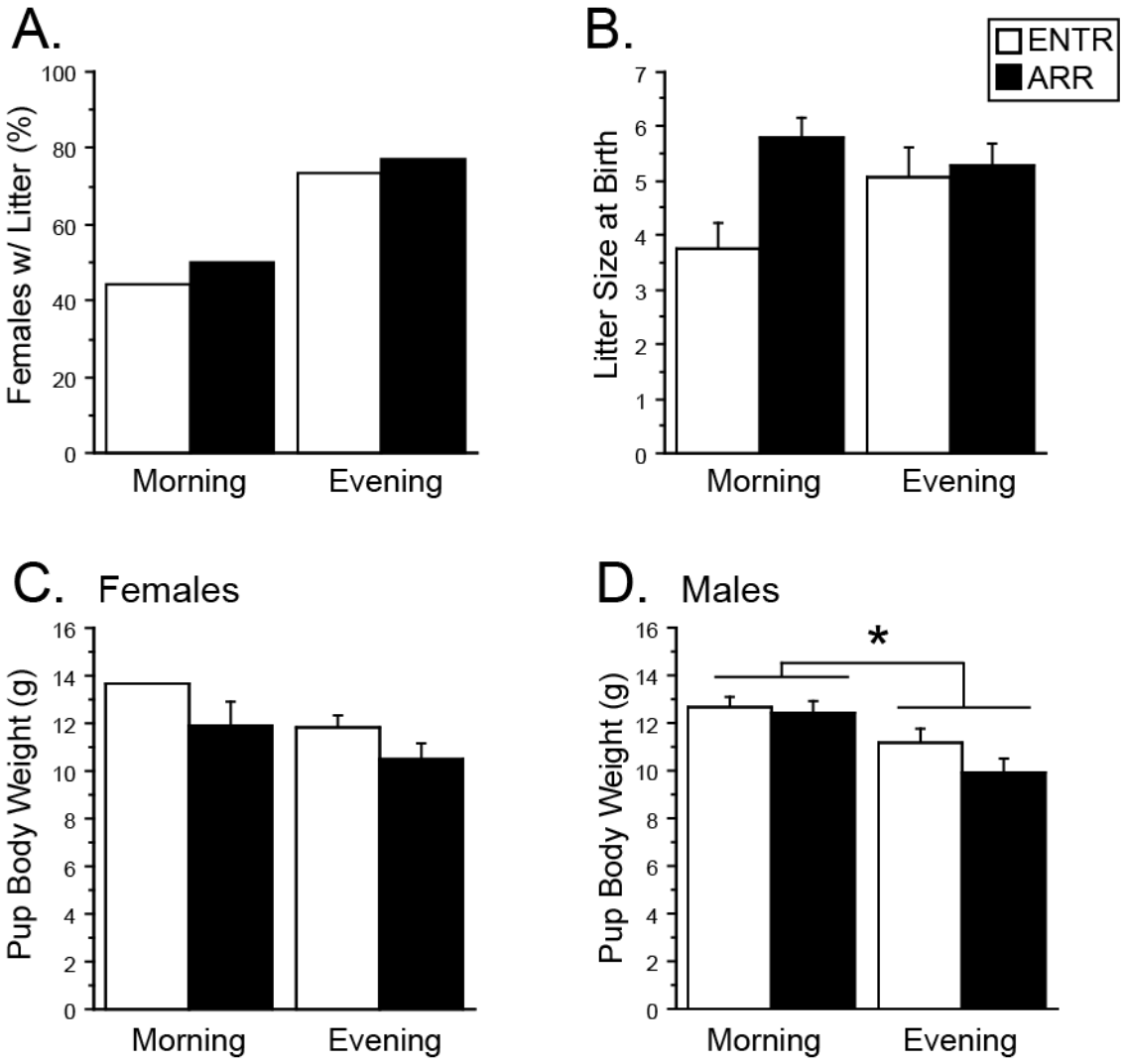


Figure 3.8. Fertility outcome following 3-h timed pairings. Chi-square analysis for (A) proportion of ENTR and ARR females having a litter following evening (ZT15-ZT18) or morning (ZT04-ZT07) mate pairings. Mean ± SEM for (B) litter size at birth, (C) female and (D) male pup weight at weaning for ENTR and ARR dams paired in the morning or evening. No effect of phenotype or pairing was evident in proportion of females pregnant. There was a trend toward smaller litter size in ENTR females mating in the morning. Male pups show lower body weight (*$p<0.05$) when dam mated in the morning compared to evening, an effect not evident in female pups ($p>0.05$).

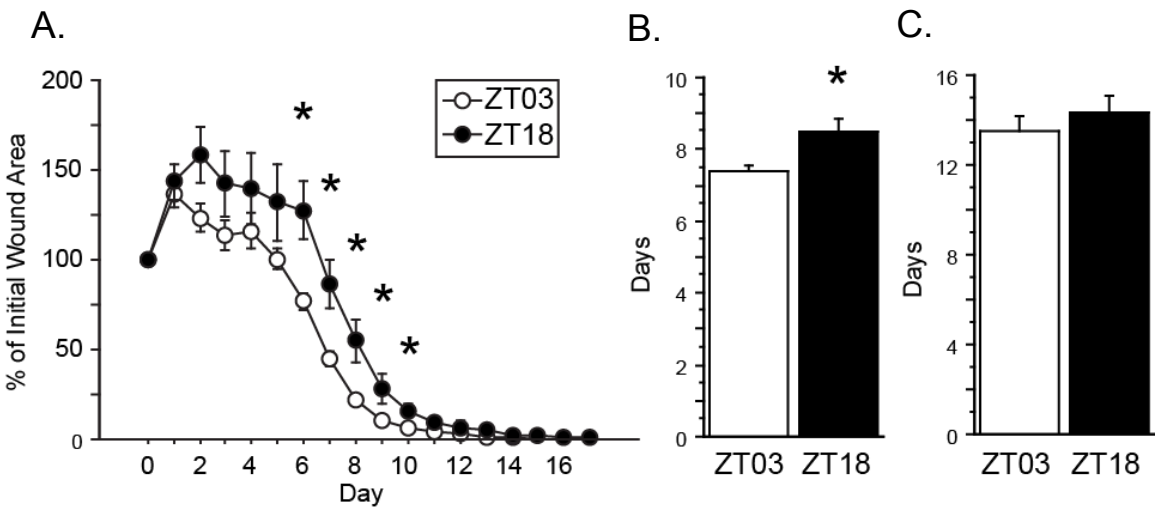


Figure 4.1. Round 1 ENTR circadian variation in wound healing. (A) Mean ± SEM of Relative Wound Size (RWS) over the full period of wound recovery in Round 1 ENTR hamsters wounded at ZT03 (open circles; n=8), and ZT18 (filled circles; n=8). Healing rate was measured by mean (±SEM) time (in days) to reach criterion for (B) 50% and (C) 100% healed in R1 ENTR hamsters wounded at ZT03 (white bars) and ZT18 (black bars). *p<0.05.

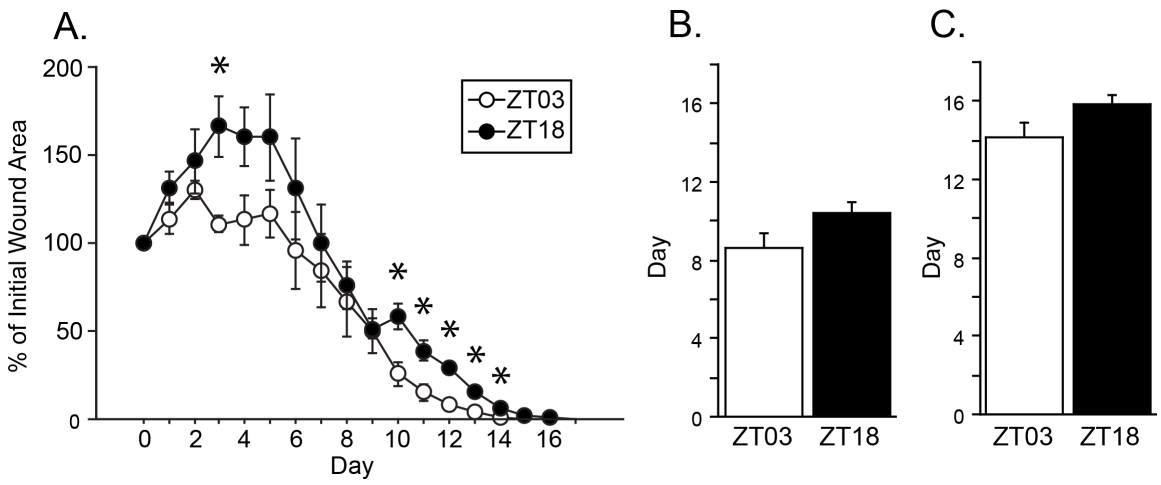


Figure 4.2. Round 2 ENTR circadian variation in wound healing. (A) Mean ± SEM of RWS over the full period of wound recovery in Round 2 ENTR hamsters wounded at ZT03 (open circles; n=5), and ZT18 (filled circles; n=5). Healing rate was measured by mean (±SEM) time (in days) to reach criterion for (B) 50% and (C) 100% healed in R2 ENTR hamsters wounded at ZT03 (white bars) and ZT18 (black bars). *p<0.05.

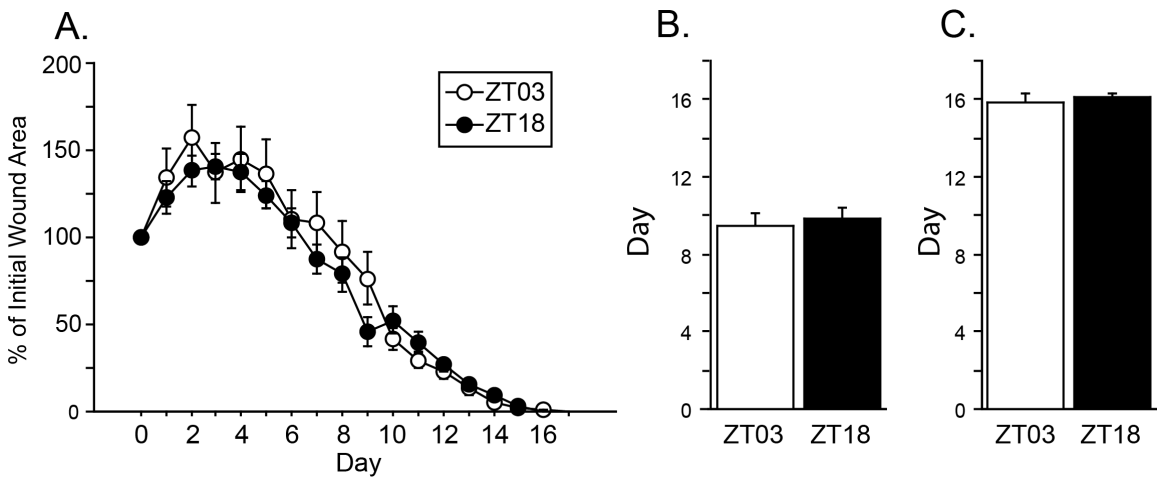


Figure 4.3. ARR circadian variation in wound healing. (A) Mean ± SEM of RWS over the full period of wound recovery in ARR hamsters wounded at ZT03 (open circles; n=8), and ZT18 (filled circles; n=6). Healing rate was measured by mean (±SEM) time (in days) to reach criterion for (B) 50% and (C) 100% healed in ARR hamsters wounded at ZT03 (white bars) and ZT18 (black bars).

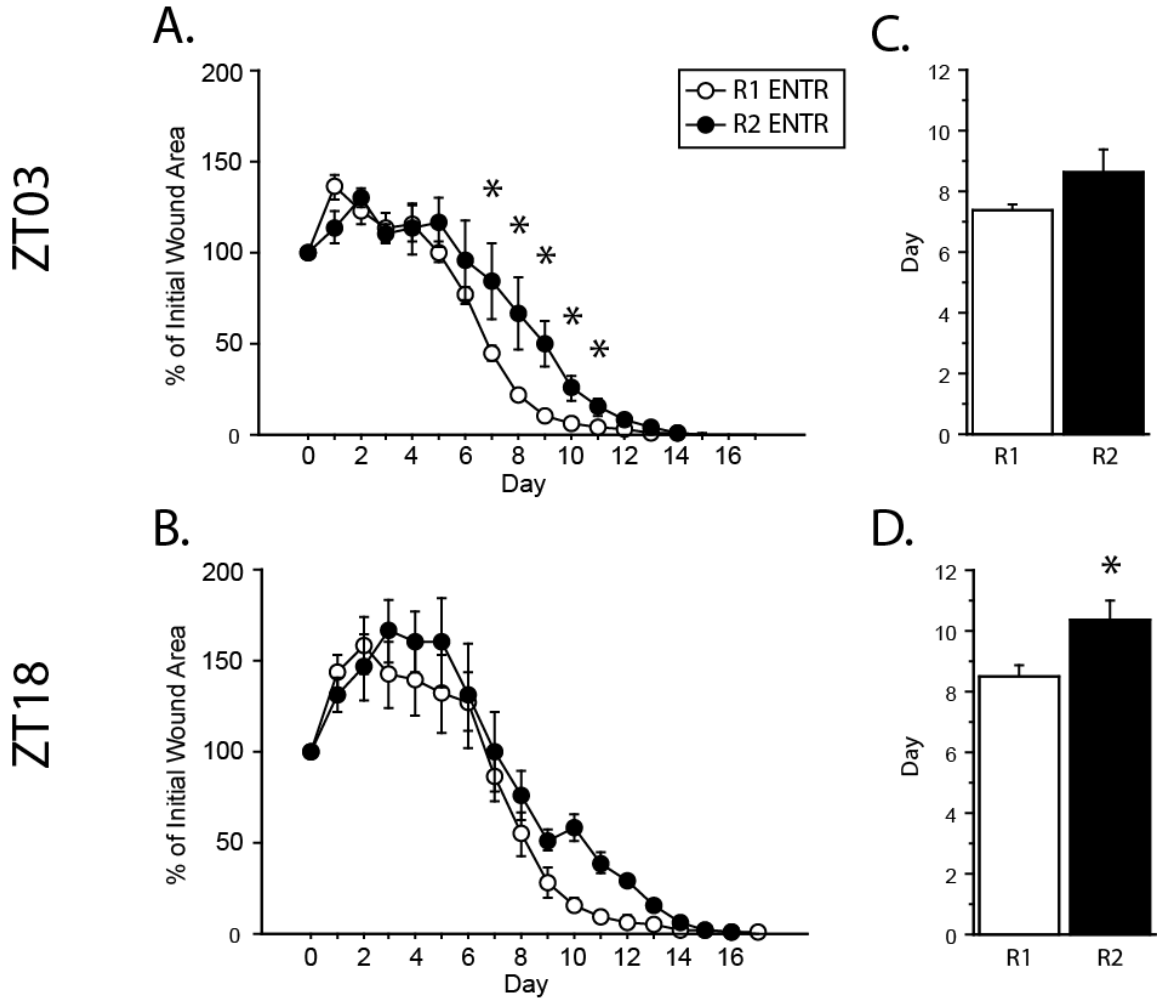


Figure 4.4. Chronotypic variation in wound healing between Round 1 ENTR and Round 2 ENTR hamsters. Mean ± SEM of RWS over the full period of wound recovery in R1 ENTR hamsters (open circles), and R2 ENTR hamsters (filled circles) wounded at (A) ZT03 and (B) ZT18. Healing rate was measured by mean (±SEM) time (in days) to reach criterion for 50% healed in R1 ENTR (white bars) and R2 ENTR hamsters (black bars) wounded at (C) ZT03 and (D) ZT18. *p<0.05.

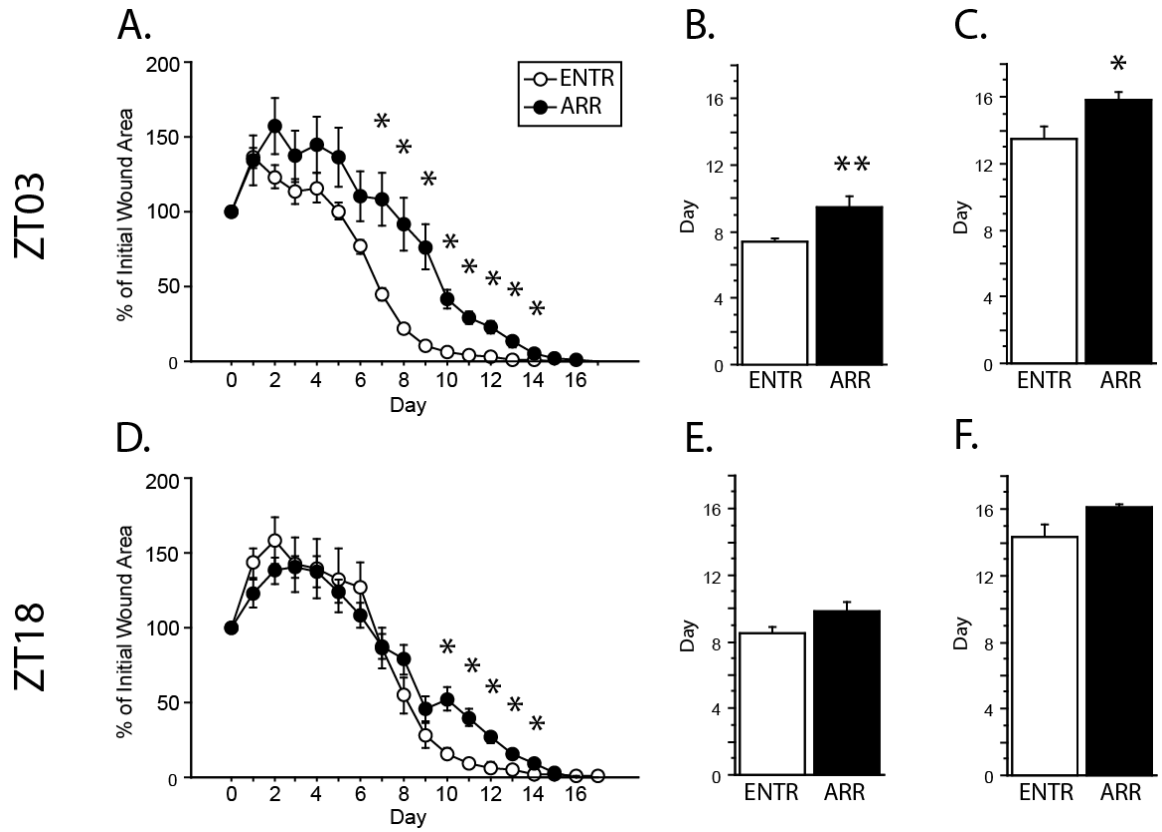


Figure 4.5. Chronotypic variation in wound healing between robustly ENTR and ARR hamsters. Mean ± SEM of RWS over the full period of wound recovery in R1 ENTR hamsters (open circles), and ARR hamsters (filled circles) wounded at (A) ZT03 and (D) ZT18. Healing rate was measured by mean (±SEM) time (in days) to reach criterion for (B) 50% and (C) 100% healed in R1 ENTR hamsters (white bars) and ARR (black bars) wounded at ZT03, and (E) 50% and (F) 100% healed in animals wounded at ZT18. *p<0.05, **p<0.01.

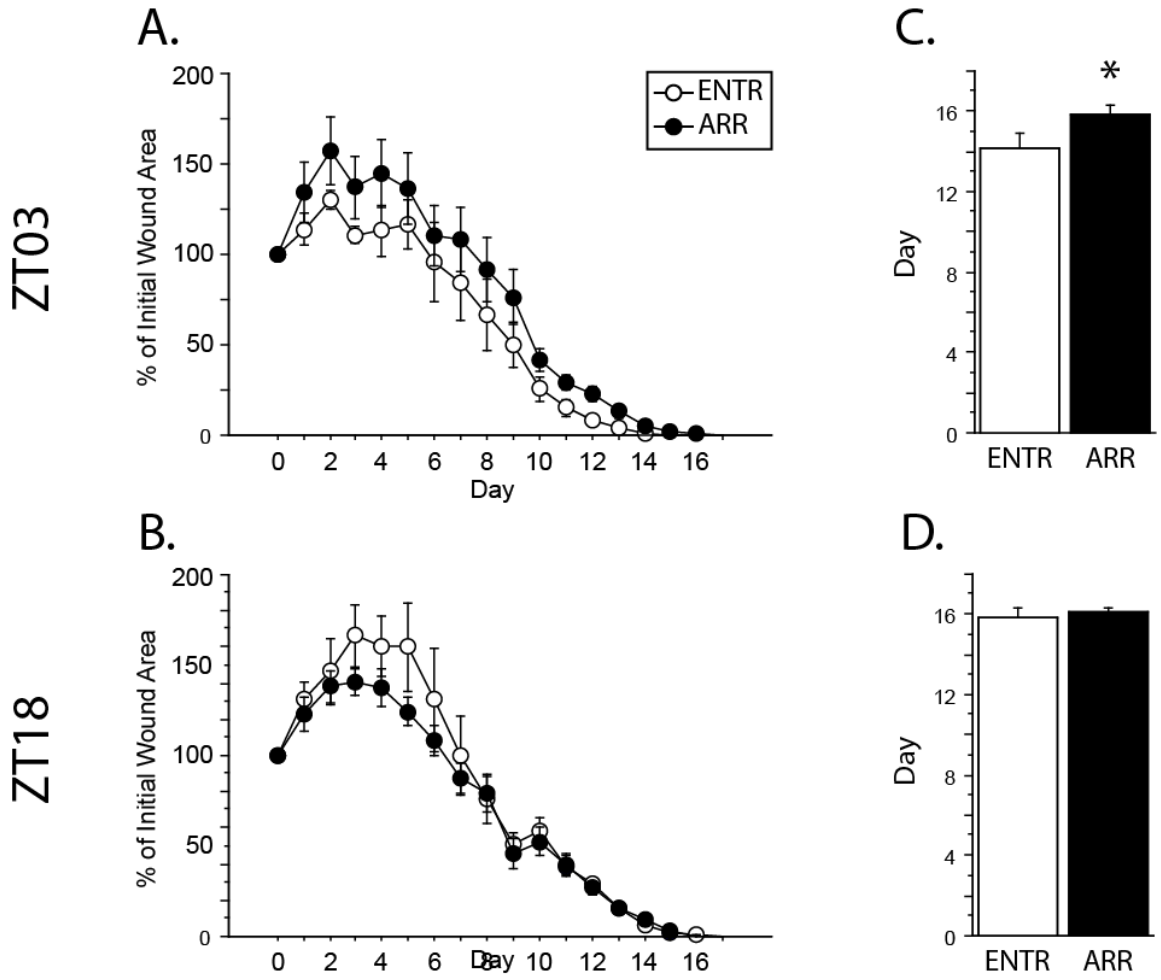


Figure 4.6. Chronotypic variation in wound healing between Round 2 ENTR and ARR hamsters. Mean ± SEM of RWS over the full period of wound recovery in R2 ENTR hamsters (open circles), and ARR hamsters (filled circles) wounded at (A) ZT03 and (B) ZT18. Healing rate was measured by mean (±SEM) time (in days) to reach criterion for healed in R2 ENTR hamsters (white bars) and ARR (black bars) wounded at (C) ZT03, and (D) ZT18. *p<0.05.

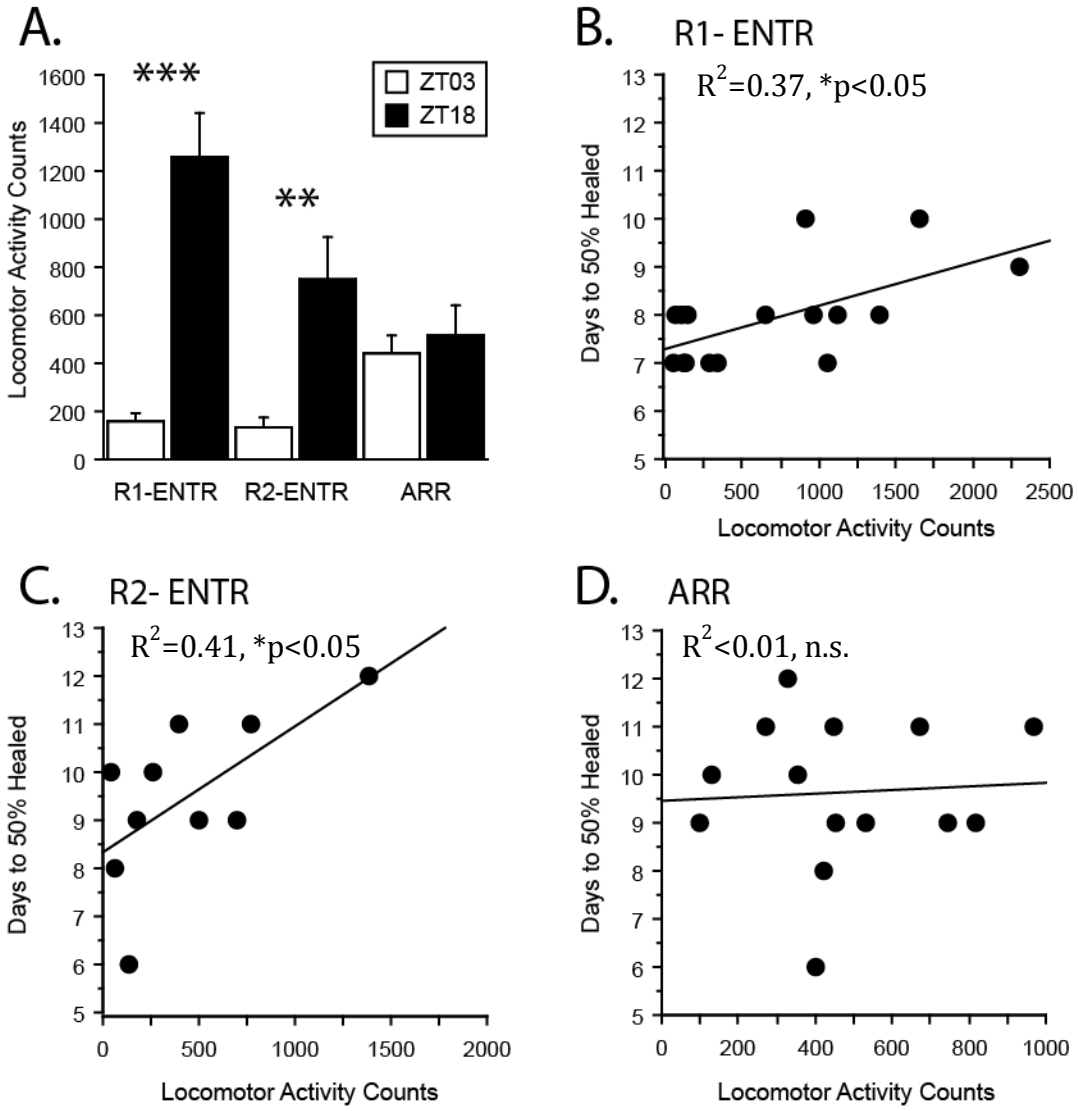


Figure 4.7. Circadian and chronotypic variation in locomotor activity. (A) Mean ± SEM differences in locomotor activity in two separate 8h epochs (ZT03-11, white bars; ZT18-02, filled bars) in Round 1 ENTR, Round 2 ENTR, and ARR hamsters wounded at ZT03 (white bars) and ZT18 (filled bars). Linear regressions assessed locomotor activity as a predictor of time to 50% wound healing in (B) Round 1 ENTR, (C) Round 2 ENTR, and (D) ARR hamsters. **p<0.01, ***p<0.001.

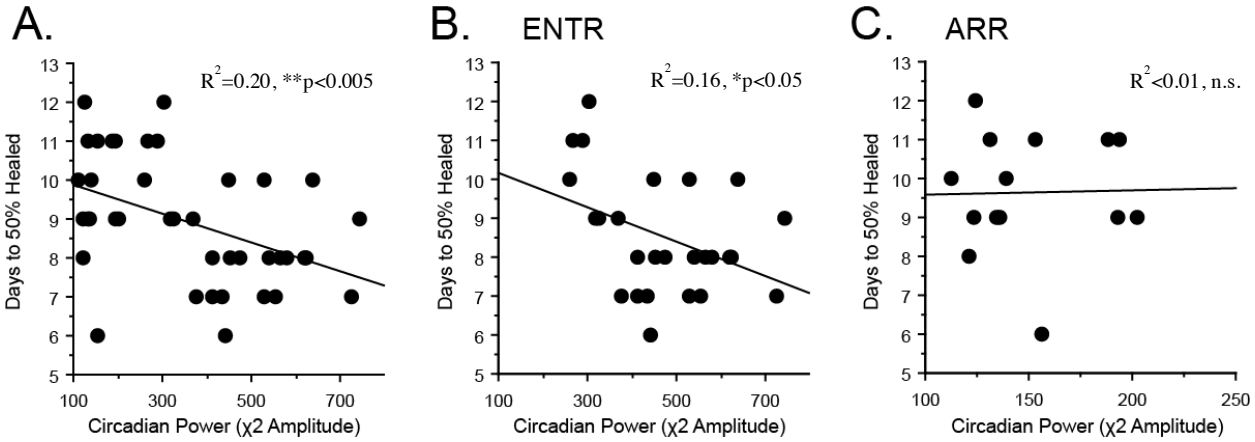


Figure 4.8. Circadian power in wound healing. (A) Linear regression for circadian power as a predictor of 50% wound healing across all phenotypes and wound times (**p<0.005). Supplementary regressions on circadian power and 50% healing were performed separately on (B) ENTR hamsters (*p<0.05) and (C) ARR hamsters (n.s. p>0.90) to negate influence of low Qp values in ARR hamsters.

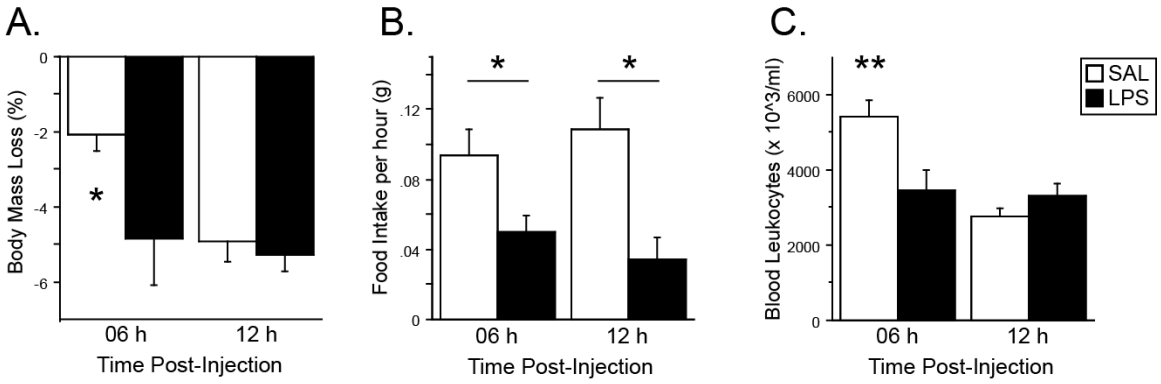


Figure 5.1. Peripheral sickness response in free-cycling female hamsters. Mean±SEM measures of (A) percent body mass change, (B) food intake per hour, and (C) blood leukocyte concentration for saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h (n=11 SAL; n=15 LPS) and 12 h (n=12 SAL; n=11 LPS) post-injection. LPS-injected hamsters had increased body mass loss and decreased food intake, compared to saline-injected females (*$p<0.05$). LPS-injected females also were significantly disrupted in leukocyte trafficking compared to saline-injected females (**$p<0.01$).

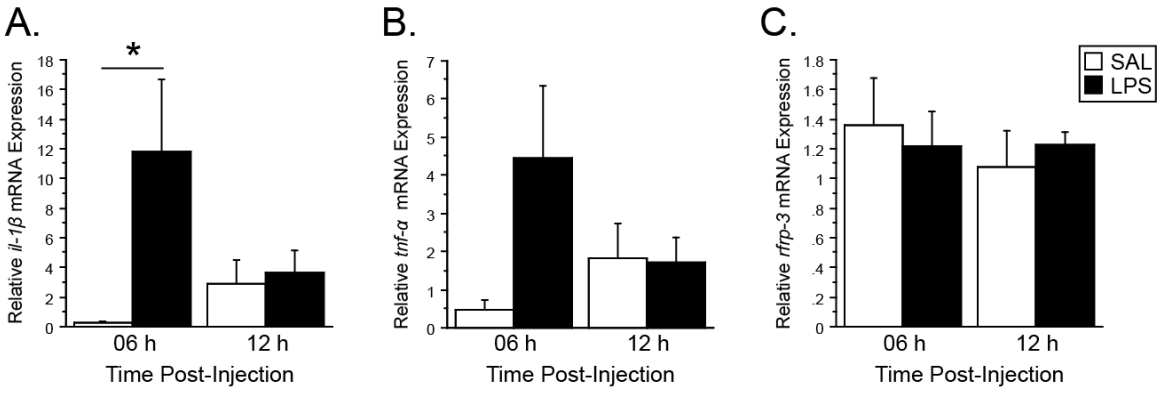


Figure 5.2. Anterior hypothalamic mRNA expression in free-cycling female hamsters. Mean±SEM relative mRNA expression for (A) *il-1β*, (B) *tnf-α* and (C) *rfrp-3* in the anterior hypothalamus of saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h and 12 h post-injection. At 6 h post injection, *il-1β* expression was elevated in LPS-injected females compared to saline-injected females (*p<0.05).

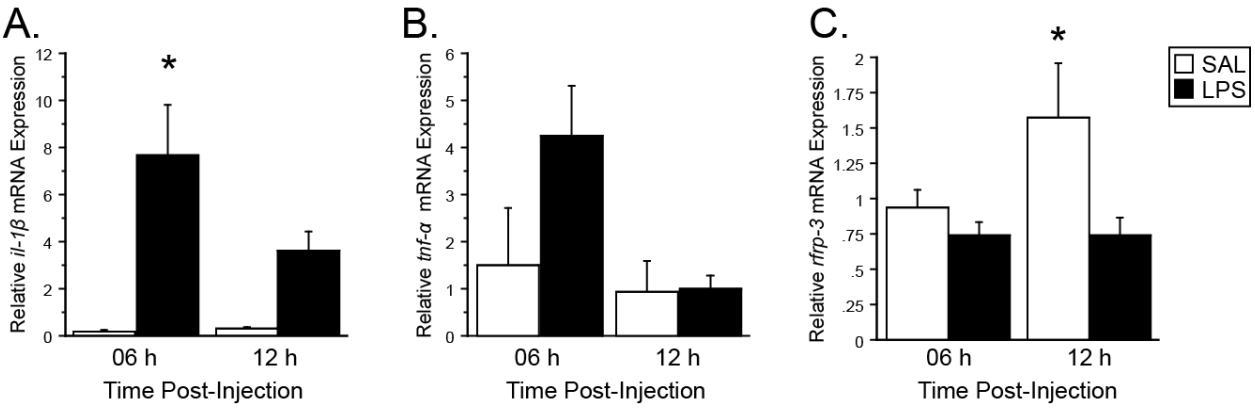


Figure 5.3. Posterior hypothalamic mRNA expression in free-cycling female hamsters. Mean±SEM relative mRNA expression for (A) *il-1β*, (B) *tnf-α* and (C) *rfrp-3* in the posterior hypothalamus of saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h and 12 h post-injection. At 6 h post injection, *il-1β* expression was elevated in LPS-injected females compared to saline-injected females (*p<0.05). *Rfrp-3* expression was decreased in LPS-injected females compared to saline-injected females at 12 h post-injection (*p<0.05).

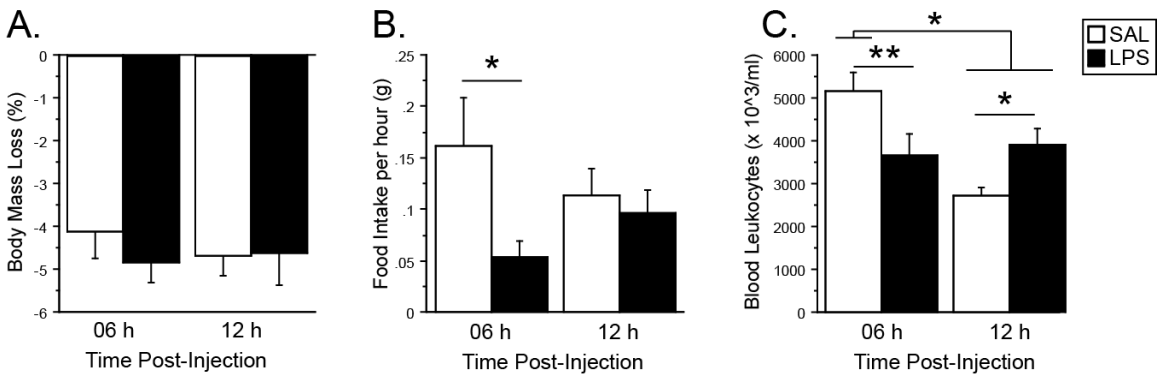


Figure 5.4. Peripheral sickness response in steroid-primed female hamsters. Mean±SEM measures of (A) percent body mass change, (B) food intake per hour, and (C) blood leukocyte concentration for saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h (n=13 SAL; n=13 LPS) and 12 h (n=13 SAL; n=13 LPS) post-injection. LPS-injected hamsters had decreased food intake compared to saline-injected females at 6 h post-injection. (*$p<0.05$). LPS-injected females also were significantly disrupted in leukocyte trafficking compared to saline-injected females at both collection times, post-injection(*$p<0.05$, **$p<0.01$).

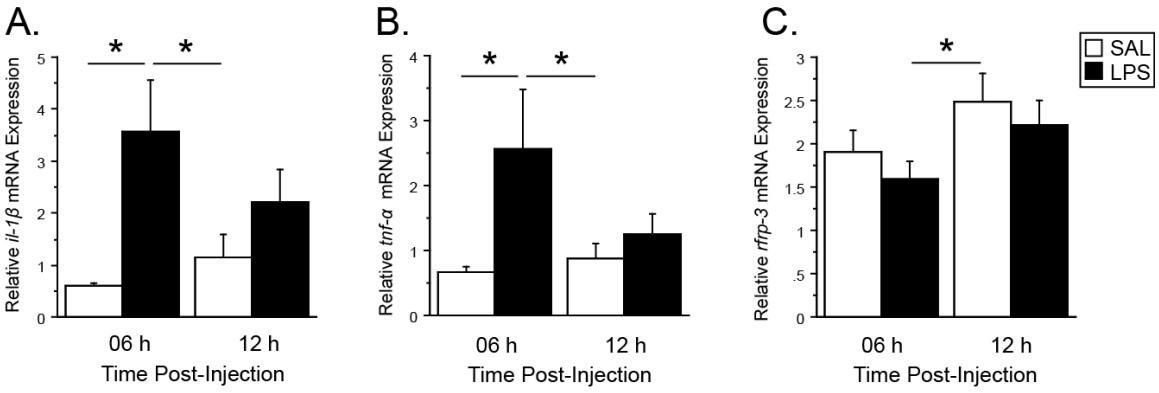


Figure 5.5. Anterior hypothalamic mRNA expression in steroid-primed female hamsters. Mean±SEM relative mRNA expression for (A) *il-1β*, (B) *tnf-α* and (C) *rfrp-3* in the anterior hypothalamus of saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h and 12 h post-injection. At 6 h post injection, *il-1β* and *tnf-α* expression was elevated in LPS-injected females compared to saline-injected females (*p<0.05, all comparisons). *Rfrp-3* was decreased in LPS-injected *rfrp-3* at 6 h post-injection, compared to *rfrp-3* in saline-injected animals at 12 h post-injection (*p<0.05).

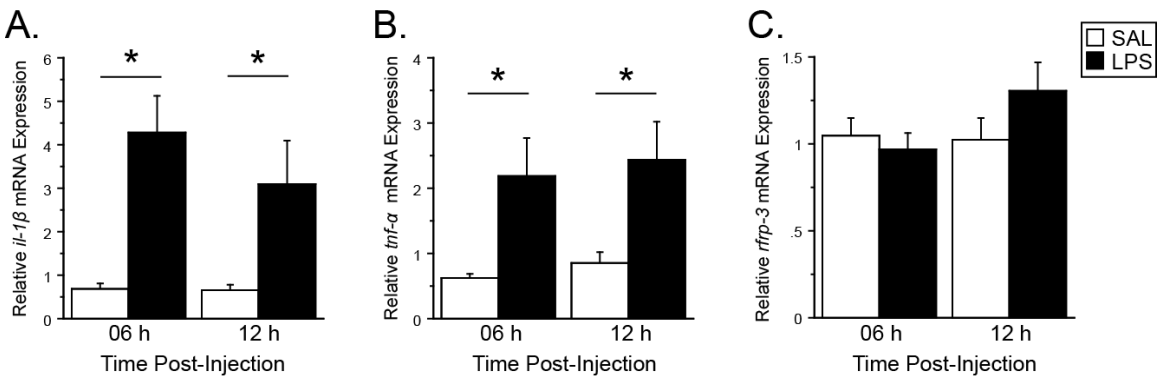


Figure 5.6. Posterior hypothalamic mRNA expression in steroid-primed female hamsters. Mean±SEM relative mRNA expression for (A) *il-1β*, (B) *tnf-α* and (C) *rfrp-3* in the posterior hypothalamus of saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h and 12 h post-injection. At 6 h post injection, *il-1β* and *tnf-α* expression was elevated in LPS-injected females compared to saline-injected females (*$p<0.05$, all comparisons).

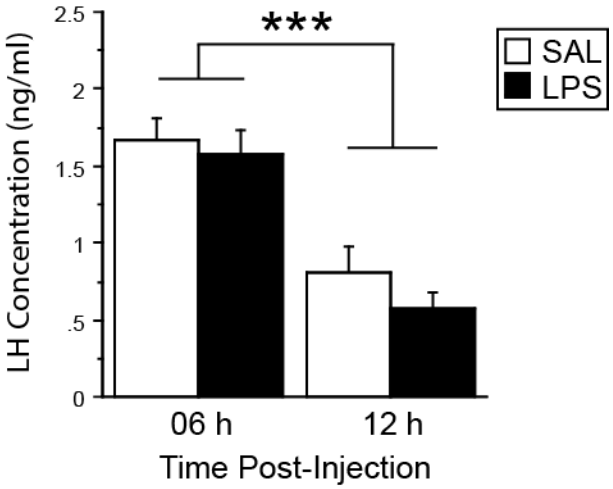
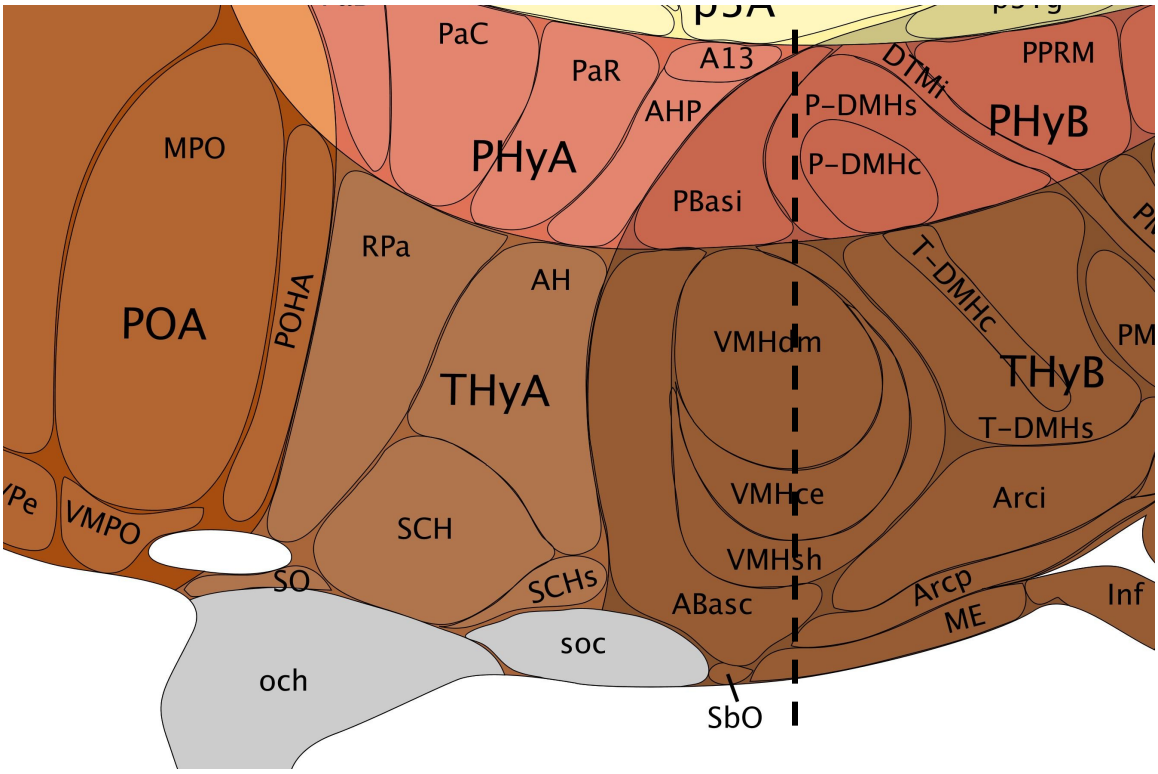
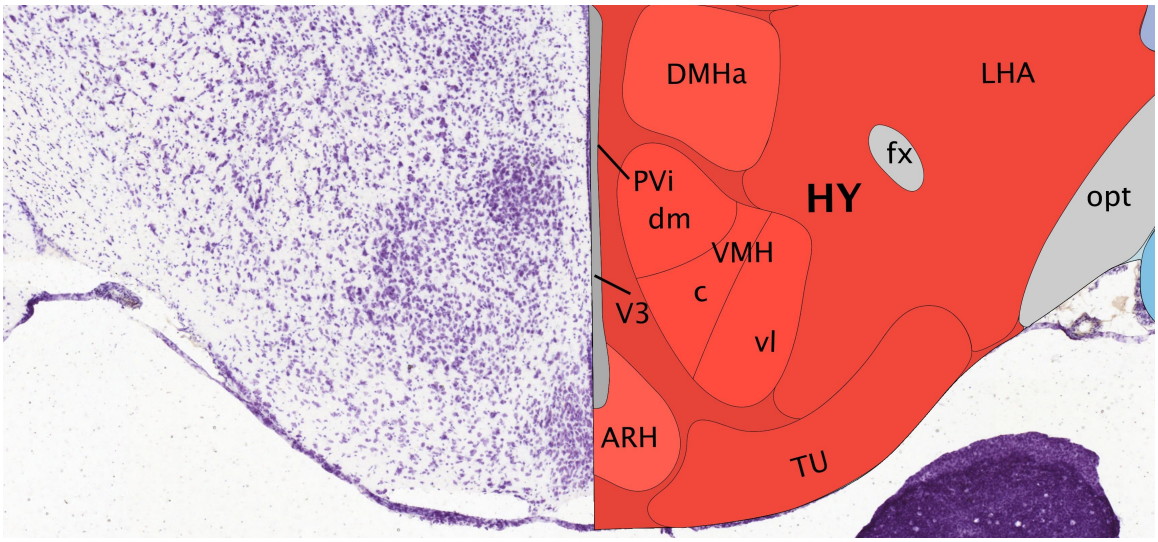


Figure 5.7. Blood LH concentration in steroid-primed hamsters. Mean±SEM concentration for circulating LH (ng/ml) in saline (SAL, white bars) and LPS-injected (LPS, black bars) hamsters at 6 h and 12 h post-injection. Both types of injection yielded increased concentration of LH at 6 h post-injection compared to 12 h post-injection (***$p<0.001$, all comparisons).



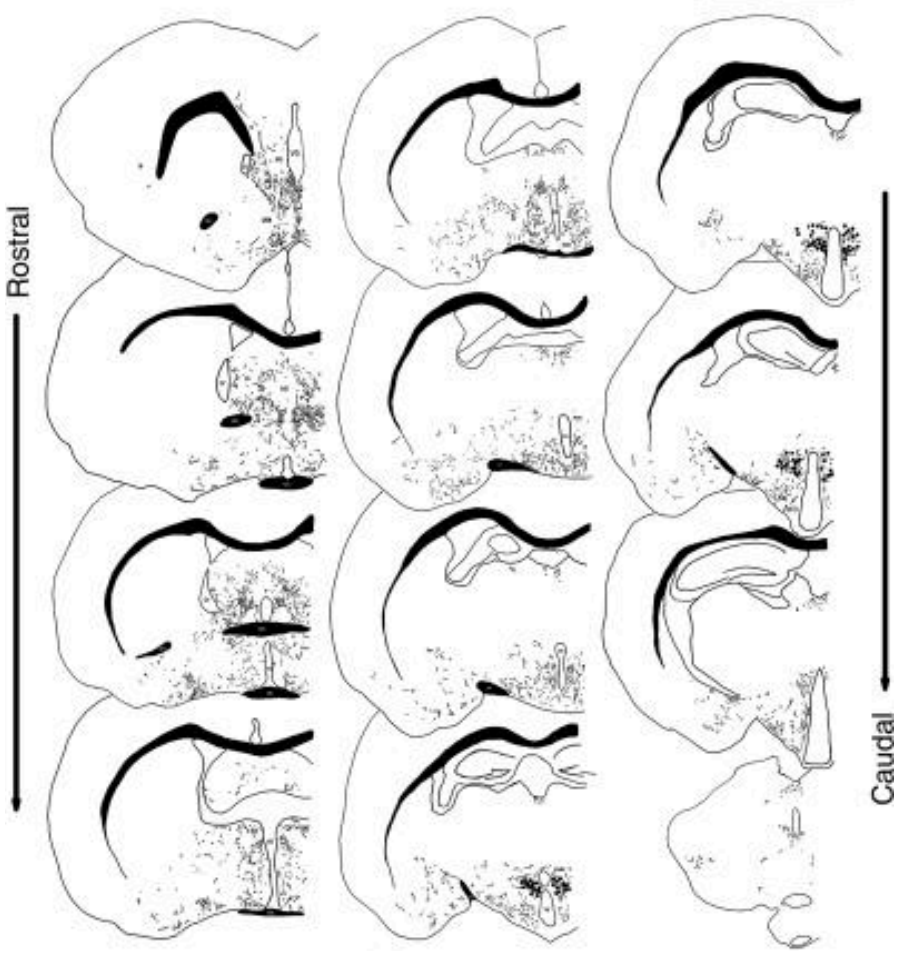
Supplementary Figure 1: Sagittal illustration of the mouse hypothalamus, with neuronal subpopulations localized. Vertical dashed line indicates approximate location of coronal incision for microdissection of anterior and posterior hypothalamus in Experiment 5.1 and 5.2. Note the bisection of the ventromedial hypothalamus (VMH), and the anterior portion of the dorsomedial hypothalamus (DMH).

Image acquired from the Allen Brain Atlas (P56 Sagittal, Image 21): http://atlas.brain-map.org/atlas?atlas=181276165#atlas=181276165&plate=100883921&structure=15662&x=7883.615234375&y=6701.5&zoom=-1&resolution=4.19&z=6



Supplementary Figure 2: Coronal illustration of mouse hypothalamus. Image visualizes the neuronal subpopulations at the approximate location of coronal incision for microdissection of anterior and posterior hypothalamus in Experiment 5.1 and 5.2. Coronal incisions were made where the optic tract (opt) was no longer visible on the dorsal surface of the hypothalamus. The ventromedial hypothalamus (VMH), and the anterior portion of the dorsomedial hypothalamus (DMHa) are both included at this location, and are bisected by the incision.

Image acquired from the Allen Brain Atlas (P56 Coronal, Image 67): http://atlas.brain-map.org/atlas?atlas=1#atlas=1&structure=125&resolution=4.65&x=5488.444281684027&y=6469.443901909722&zoom=-2&plate=100960256&z=6



Supplementary Figure 3: RFRP-3 cell bodies and projections in the Syrian hamster brain. Illustration of rostral to caudal sagittal sections stained for RFRP-3 cell bodies and fiber projections throughout the hamster brain. Incisions made during hypothalamic microdissections in Experiment 5.1 and 5.2 would have been localized approximately at the top right section.

Image acquired and adapted from Kriegsfeld et al. (2006).